

**EPIGENETIC REGULATION OF SILENT CHROMATIN IN *Saccharomyces*
*cerevisiae***

A Dissertation

by

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ABSTRACT

The ribosomal DNA (rDNA) locus in *Saccharomyces cerevisiae* contains 150-200 tandem repeats of the ribosomal RNA genes. The rDNA acquires a special chromatin structure called silent chromatin that represses RNA Polymerase (Pol) II transcription and homologous recombination to protect the integrity of this ~1.8 Mb locus.

To better understand rDNA silent chromatin, sixteen yeast strains, each with a single Pol II-transcribed *HIS3* reporter gene in a different rDNA repeat unit, were analyzed to compare silent chromatin in individual rDNA repeats across the rDNA array. Experiments comparing the level of *HIS3* mRNA and the rate of recombination at the rDNA revealed that chromatin within individual rDNA repeats does not equally silence Pol II transcription and recombination. Repeat-specific variation in the strength of silent chromatin is called rDNA Position Effect. Chromatin immunoprecipitation experiments showed that the association of Sir2, a protein required for silent chromatin, varies at the *HIS3* promoter; more Sir2 is associated with more silent rDNA repeats. The mechanism by which the association of Sir2 varies has not yet been identified. Until now, rDNA silent chromatin was assumed to be uniform across the rDNA array; the discovery of rDNA Position Effect alters this basic understanding and indicates rDNA silent chromatin is dynamic.

For further insight, a Tet-Off system is being developed to control the expression of Sir2. Depleting Sir2 by the addition of doxycycline to growth media compromises

rDNA silent chromatin; removal of doxycycline allows for Sir2 to accumulate and rDNA silent chromatin to re-establish. Once optimized, this Tet-Off system will be used in experiments to investigate the establishment of rDNA silent chromatin in a single rDNA repeat unit by controlling the level of Sir2 in the cell.

Finally, spot plate growth analysis has revealed that cells lacking the histone methyltransferase Set1 are sensitive to the herbicide 3-Amino-1,2,4-triazole. Preliminary data suggest this new phenotype is due to the loss of methylation of lysine four of the histone H3 tail (H3K4). Assessment of mRNA levels supports a new role for H3K4 methylation in stress-response transcriptional regulation.

This research has increased the fundamental understanding of the conserved epigenetic regulation of chromatin.

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I. INTRODUCTION

The regulation of transcription is an essential activity of the cell, and the structure of DNA plays a key role in this process. In eukaryotic cells, DNA is wrapped around a core octamer of histones, two copies each of the histones H2A, H2B, H3, and H4. This nucleosome forms the basis for the packaging of DNA into a highly ordered structure called chromatin [1]. Amino acid sequences that protrude from the core of the histone proteins are called histone tails. Many types of modifications can be made to the histone tails, and these modifications have been implicated in many ways to regulate transcription by changing the landscape of chromatin structure, in turn dictating the association of chromatin remodeling complexes, the positioning of nucleosomes, and the association of transcription factors.

Epigenetics is the field of study aimed at understanding the regulation of chromatin and gene expression above the DNA sequence. Chromatin can be categorized as transcriptionally active euchromatin or transcriptionally inactive heterochromatin. Each type of chromatin is associated with specifically modified (or unmodified) histones that control the transcription activity of individual genes or the structure of larger regions of the genome; when regions of the genome are formed into heterochromatin, these regions are known as silent chromatin. While many studies focus on histone modifications and chromatin structure in euchromatin, the regulation of heterochromatin is also critical to the viability of a cell. Repeated DNA sequences are often found in heterochromatic states in order to preserve the integrity of the genome because repeated

sequences are prone to be rearranged by homologous recombination [2, 3].

Misregulation of repeated DNA sequences can lead to cell death and can be detrimental to an organism. With half or more of the human genome made up of repetitive sequences [4], it is imperative to have a more thorough understanding of how repeated DNA sequences are epigenetically regulated.

The ribosomal DNA (rDNA) arrays of organisms from yeast to human are found as tandem head-to-tail repeats, although the content and number of repeat clusters varies [3, 5, 6]. In humans, the approximately 350 copies of the rRNA genes are found in five clusters on five different chromosomes; genes encoding the 45S precursor rRNA are in tandem repeats on chromosomes 13, 14, 15, 21, and 22. In contrast, the rDNA array in *S. cerevisiae* is found as a single array on chromosome (chr) *XII*, and each of the 150-200 tandem repeats includes both the 35S and 5S rRNA genes. Despite the difference in organization, approximately half of the rDNA repeats in both organisms are silenced [5, 7, 8].

Regulation of rDNA arrays is important not only for genome stability but also because growth of the cell is directly related to ribosome biogenesis. When the need arises for a cell to change the rate of protein synthesis it alters the number of ribosomes in the cell; this starts with regulating transcription of the rRNA genes [5, 9, 10]. In addition, changes in rDNA chromatin structure, stability and regulation have been associated with aging and neurodegenerative diseases, such as Alzheimer's and Parkinson's, adding to the significance of understanding the foundation of rDNA regulation [11-13].

The budding yeast *Saccharomyces cerevisiae* is a powerful model organism for studies to gain a better understanding of the epigenetic regulation of rDNA repeats. Transcription of reporter genes integrated into the rDNA can be used as a measure of rDNA silent chromatin. In contrast to higher eukaryotes, *S. cerevisiae* shows no evidence of DNA methylation or RNA interference, simplifying the study of conserved histone modifications and other regulatory mechanisms because the effects on transcript levels can be directly assessed [14, 15]. This dissertation focuses on understanding the epigenetic regulation of silent chromatin at the rDNA locus of *S. cerevisiae*.

1.1 THE RIBOSOMAL DNA IN *S. cerevisiae*

The rDNA locus in *S. cerevisiae* is made up of 150-200 identical tandem repeat units on chr *XII*. Each repeat unit is 9.1 kilobases in length, making the entire rDNA array ~1.8 megabases. Contained within a single repeat unit is the RNA Polymerase (Pol) I transcribed 35S rRNA gene, the Pol III transcribed 5S rRNA gene, and a non-transcribed spacer (NTS) that is divided into two regions, NTS1 and NTS2, by the 5S rRNA gene (Figure 1-1). Several *cis*-regulatory elements are found within the non-transcribed spacers. Within NTS2 is an origin of DNA replication; about 20% of these fire during each cell cycle [16, 17]. Non-coding RNAs can be transcribed by Pol II from non-coding transcription units in NTS2 [18]. Each NTS1 region has a replication fork barrier element, which prevents bidirectional replication from interfering with Pol I transcription [19, 20]. Fob1, a protein important for recombination and silencing in the rDNA, binds the replication fork barrier [21, 22]. Also in NTS1 is E-pro, a bidirectional

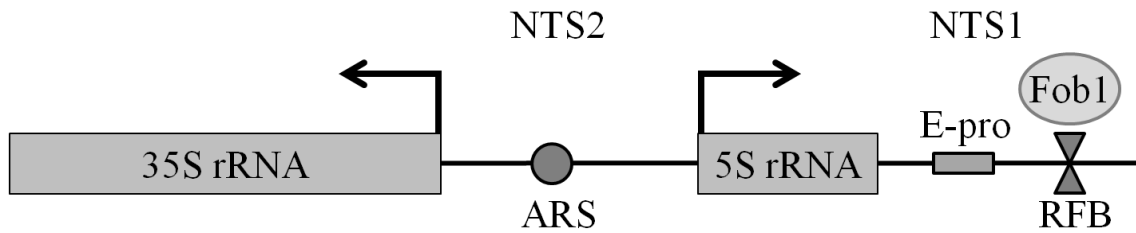


Figure 1-1. Diagram of a single rDNA repeat unit. Each of the 150-200 tandem rDNA repeat units are organized as shown. Arrows indicate the direction of Pol I (35S rRNA) and Pol III (5S rRNA) transcription. ARS, origin of replication (autonomous replicating sequence). RFB, replication fork barrier. E-pro, Pol II promoter that produces non-coding RNAs. The protein Fob1 binds to the RFB. NTS1, non-transcribed spacer 1; NTS2, non-transcribed spacer 2.

Pol II promoter that produces non-coding RNAs. Silencing of E-pro has a proposed role in maintaining rDNA copy number [23-25].

The rDNA array acquires silent chromatin structure; Pol II transcribed genes are silenced when integrated into the rDNA despite active transcription from Pol I and Pol III [26-28]. rDNA silent chromatin is important for preserving the integrity of the highly repeated rDNA locus because inactive chromatin is less apt to participate in inappropriate homologous recombination between rDNA repeat units [29]. When silent chromatin is lost, the rate of mitotic recombination increases, which destabilizes this large portion of the genome. Transcription by Pol II of reporter genes and non-coding RNAs in the NTS is also increased when silent chromatin is compromised [18, 24].

1.2 REGULATION OF rDNA SILENT CHROMATIN IN *S. cerevisiae*

1.2.1 Sir2 and the RENT complex

Silencing proteins and modified histones are critical components of silent chromatin in *S. cerevisiae*. Although many proteins have been identified as being regulators of silent chromatin, the Silent Information Regulator (SIR) proteins are required for the formation and function of silent chromatin at the three known silent chromatin regions of the *S. cerevisiae* genome. The requirement for specific SIR proteins varies at each silent domain (see Section 1.4).

Sir2 is an NAD-dependent histone deacetylase responsible for deacetylation of lysine residues in histone tails, including lysines 9 and 14 in histone H3 (H3K9 and H3K14) in *S. cerevisiae* [30-33]; it is conserved in higher eukaryotes [34]. Sir2 was one

of the first proteins shown to regulate silent chromatin at the rDNA array [26-28]. Although additional SIR proteins have a role in silent chromatin at the other silent domains in *S. cerevisiae* (see Section 1.4), Sir2 is the only SIR protein to act in rDNA silent chromatin. The histone deacetylase activity of Sir2 is required to prevent the accumulation of acetylated histone H3 and the association of Pol II within the rDNA locus [18, 35]. The level of Sir2 protein is limiting in the yeast cell, and the silencing capacity, or strength, of silent chromatin at the rDNA has been shown to respond directly to the level of expression of Sir2 in the cell. When the level of Sir2 in cells is reduced, rDNA silent chromatin is compromised, and when Sir2 is overexpressed, rDNA silent chromatin is more repressive [36].

Sir2 is one of three proteins that make up the rDNA-silencing complex known as RENT (REgulator of Nucleolar silencing and Telophase exit). Net1 is a component of RENT that functions solely at the rDNA to recruit Sir2 to specific regions of the rDNA repeat unit [37]. Cdc14 is the final RENT member; it is sequestered in the nucleolus in order to regulate exit from mitosis, but its role in regulating silent chromatin at the rDNA has not been characterized [38].

The RENT complex interacts directly with the rDNA locus. Chromatin immunoprecipitation experiments have shown that Sir2 and Net1 associate with two regions of the rDNA repeat unit, in NTS1 at the replication fork barrier and in NTS2 at the promoter region of the 35S rRNA gene (Figure 1-2) [22]. Association of the RENT complex with the 35S rRNA promoter occurs through direct interaction between Net1 and Pol I [39]. Association of RENT in NTS1 at the replication fork barrier is through

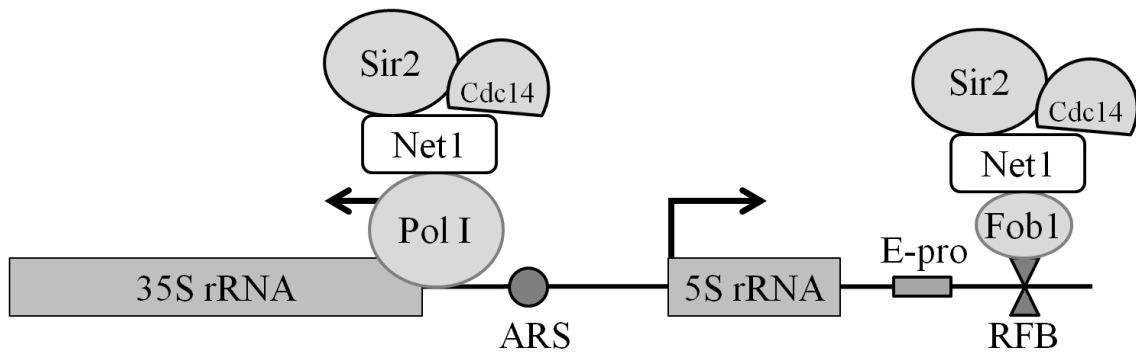


Figure 1-2. The RENT complex associates with two regions of the rDNA repeat unit. Net1 interacts with Pol I and Fob1, and is responsible for recruiting Sir2 to form silent chromatin across the rDNA array. Abbreviations, as in Figure 1-1.

interaction of Net1 with Fob1 (Figure 1-2) [22]. When either the *SIR2* or *NET1* gene is deleted from a yeast cell, silent chromatin does not form at the rDNA and the resulting high levels of Pol II transcription and homologous recombination contribute to the instability of the locus to the detriment of the cell [25, 40].

1.2.2 Set1 and the rDNA

Methylation of histone H3 on lysine 4 (H3K4) is another epigenetic mark that influences rDNA silent chromatin. The enzyme responsible for H3K4 methylation in *S. cerevisiae* is Set1 [41]. Set1 is a member of the eight-member COMPASS complex [42]. It is the only H3K4 methyltransferase in *S. cerevisiae*, and it is responsible for mono-, di- and tri-methylation of H3K4 [41]. Set1 and the COMPASS complex are highly conserved; MLL1 in humans is a histone lysine methyltransferase that is also capable of mono-, di-, and tri-methylation of H3K4 [43]. MLL1 is implicated in leukemia; however, elucidating the role of MLL1 in cell lines is complicated because there are multiple H3K4 methyltransferases in mammalian cells, making study of Set1 in *S. cerevisiae* a valuable tool for better understanding the Set-domain family of proteins.

Set1 is required for Pol II gene silencing at the rDNA; loss of Set1 activity results in a three-fold increase in Pol II transcription in the rDNA. However, unlike Sir2, Set1 does not contribute to the repression of homologous recombination at the rDNA [35, 41]. Although genetic interaction studies suggest that Set1 and Sir2 act independently to regulate silent chromatin at the rDNA [35], Sir2 has been shown to prevent the accumulation of K4-di- and tri-methylated histone H3 at the rDNA [18]. The mechanism through which Set1 regulates silent chromatin at the rDNA requires its

catalytic activity, but how low levels of K4-methylated H3 contribute to silent chromatin at the rDNA remains unknown.

1.2.3 Pol I activity and rDNA silencing

In addition to silencing proteins and histones, transcription of the 35S rRNA genes by Pol I also contributes to silent chromatin at the rDNA. A low level of basal transcription of the 35S rRNA genes requires Pol I, Rrn3 and core factor (Rrn6, Rrn7, and Rrn11), while higher levels of transcription are stimulated by the binding of the upstream activating factor (UAF; Rrn5, Rrn9, Rrn10, Uaf30) and TATA binding protein (TBP) to the upstream activating sequence (Figure 1-3) [44-50]. When Pol I is inactive, either due to the loss of essential subunits or due to the loss of subunits in core factor or UAF, Pol II is able to transcribe the 35S rRNA genes at a low level from a cryptic promoter [51, 52].

Transcription by Pol I has been shown to be required for Pol II silencing in the rDNA. Silencing of a Pol II reporter gene in the rDNA is reduced in yeast strains with inactive Pol I due to deletion of the essential Pol I subunit Rpa135 or due to deletion of UAF subunits [51-53]. In another study, levels of non-coding RNA (ncRNA) transcribed from the NTS were compared in cells lacking Pol I activity due to loss of different required factors: *rrn7Δ* (core factor), *rrn5Δ* (UAF), or the essential Pol I subunit A43. Compared to a wild type yeast strain with active Pol I, levels of ncRNAs were increased in all three yeast strains with inactive Pol I, with the highest levels coming from noncoding Pol II transcription units in NTS2 [54].

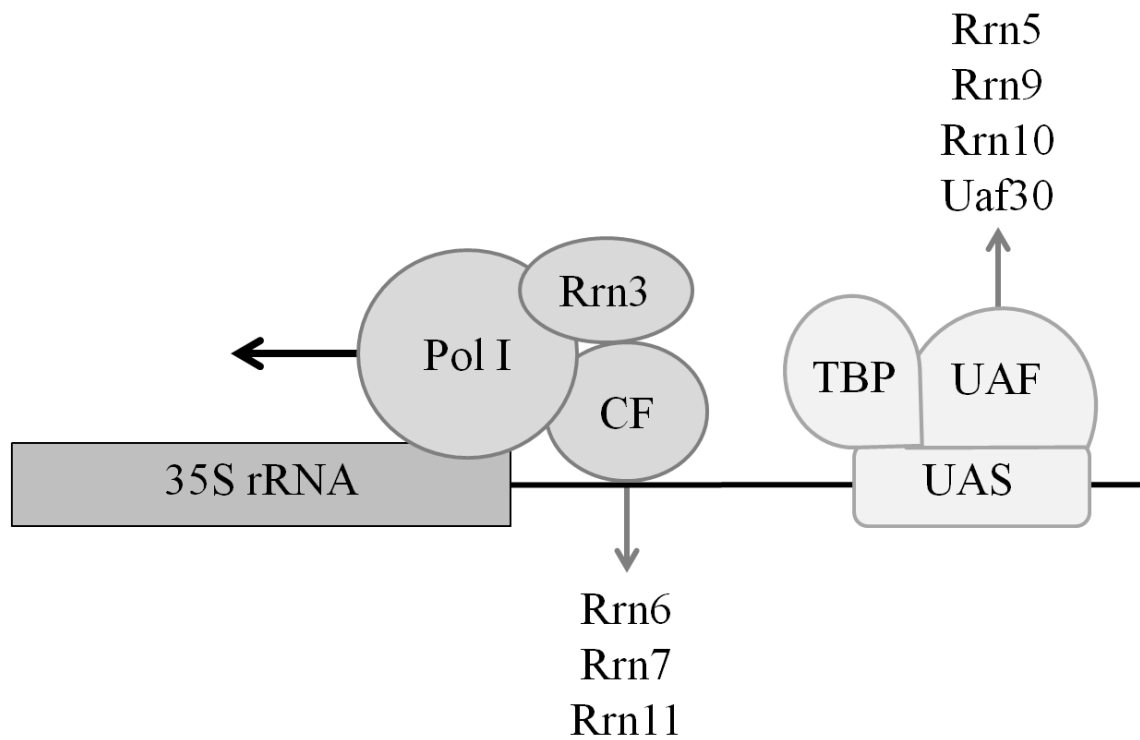


Figure 1-3. Factors required for Pol I transcription of the 35S rRNA gene.

Transcription of the 35S rRNA gene requires Pol I in complex with Rrn3 and the Core Factor (CF), comprised of Rrn6, Rrn7, and Rrn11. The upstream activating sequence (UAS) is the binding site for TATA binding protein (TBP) and the upstream activating factor (UAF). UAF is comprised of Rrn5, Rrn9, Rrn10 and Uaf30. TBP and UAF are required for Pol I transcription above a low basal level. Black arrowhead indicates direction of Pol I transcription of the 35S rRNA gene.

The requirement for Pol I activity in Pol II silencing is further supported by studies analyzing silencing in yeast strains with a reduced number of rDNA repeats, suggesting that the density of Pol I transcription complexes regulates rDNA silent chromatin. In strains with shortened rDNA arrays containing only 25 rDNA repeat units, each of the 35S rRNA genes is transcribed by Pol I to maintain viability of the cell; a reporter gene in cells with a shortened rDNA locus was more strongly silenced than in cells with a normal rDNA array of 150-200 repeat units [55]. Taken together, these studies indicate that Pol I activity reduces Pol II transcription in the rDNA array.

1.2.4 Three-dimensional organization of the rDNA

The rDNA resides in the nucleolus, a sub-nuclear body of the nucleus. The three-dimensional organization of the nucleus in *S. cerevisiae* has been shown to contribute to the regulation of silent chromatin at the rDNA. Two protein complexes, cohibin and CLIP (chromosome linkage inner nuclear membrane proteins), act together with Tof2 and Fob1 to tether the rDNA to the inner nuclear membrane (Figure 1-4) [56, 57].

CLIP is comprised of two inner nuclear membrane proteins, Heh1 and Nur1 [56]. The inner nuclear membrane proteins in CLIP have been shown to contribute positively to the stability of the rDNA locus through interactions with cohibin [56]. Cohibin contains two copies of Lrs4 and two homodimers of Csm1; Csm1 directly interacts with Tof2 [57, 58]. Tof2 interacts with Fob1, and Fob1 interacts with Net1, a member of the RENT complex that is responsible for recruiting Sir2; both Tof2 and Fob1 are associated in NTS1 at the replication fork barrier. Fob1 is required for the recruitment of Tof2 and

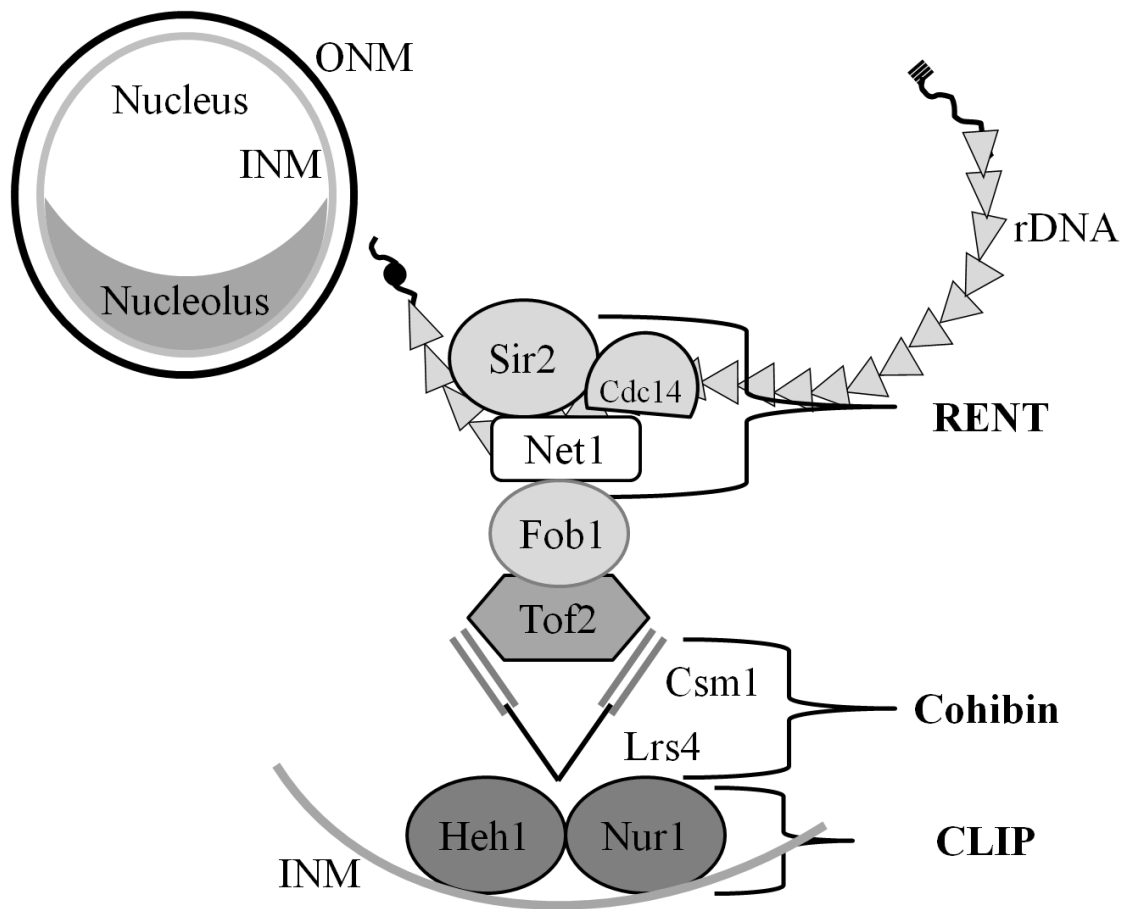


Figure 1-4. A network of proteins tethers the rDNA to the inner nuclear membrane (INM). In the nucleus, the rDNA array is sequestered in the nucleolus. CLIP, chromosome linkage INM proteins, Heh1 and Nur1. Cohibin is made up of two Lrs4 proteins and two Csm1 homodimers. Csm1 interacts with Tof2. Tof2 is recruited to NTS1 by Fob1. The RENT complex is also associated with Fob1. Together, these proteins anchor the rDNA to the INM, stabilizing the rDNA array and repressing recombination and Pol II transcription within the rDNA repeats. ONM, outer nuclear membrane.

RENT to NTS1 [22, 57].

Lrs4 and Csm1 each regulate Pol II gene silencing in NTS1, but not in NTS2. Loss of either Lrs4 or Csm1 results in an increase in unequal sister chromatid exchange; when Sir2 is deleted from *lrs4*Δ or *csm1*Δ cells, there is an additive increase in the rate of unequal sister chromatid exchange, suggesting that Sir2 and cohibin act independently to repress recombination at the rDNA [57].

Microscopy analysis of the rDNA indicates that the CLIP and cohibin proteins are required to tether the rDNA to the inner nuclear membrane and maintain a distinct nucleolar region in the nucleus. Loss of CLIP proteins Heh1 and Nur1 both result in increased unequal sister chromatid exchange. An additive increase in unequal sister chromatid exchange was observed when Sir2 was deleted from *heh1*Δ cells, suggesting that Sir2 may act independently of CLIP in repressing recombination, similar to what was found with cohibin [56, 57]. In contrast to cohibin, loss of CLIP proteins does not affect transcriptional silencing of a reporter gene in NTS1 [56].

It is interesting to consider that the three-dimensional structure of the ribosomal DNA locus formed by its tethering to the inner nuclear membrane may introduce spatial constraints that limit the interaction of the locus with silencing proteins and transcription factors, although these specific interactions have not yet been analyzed. However, tethering of the rDNA to the inner nuclear membrane does reduce the interaction of the rDNA with recombination proteins. Rad52 is known to associate with DNA double stranded breaks and a fluorescently tagged Rad52 (Rad52-YFP) can be used as a marker for visualizing sites of homologous recombination induced by DNA double stranded

breaks [59, 60]. In cells where tethering of the rDNA is abolished by deleting *HEH1* or *LRS4*, Rad52-YFP signal is located further from the nucleolus than in cells with the rDNA tethered to the inner nuclear membrane, where the Rad52-YFP signal is found at the edge of the nucleolus [56]. Additionally, when the 35S and 5S rRNA are expressed from multicopy plasmids, thereby reducing the structure of the nucleolus, Rad52-YFP foci were more dispersed than in cells with a normal rDNA array [61]. It is not unreasonable to hypothesize that the structure of the rDNA formed by tethering to the inner nuclear membrane could dictate the strength of silencing in different rDNA repeat units by spatially dictating what and how many silencing proteins can associate with a particular rDNA repeat or region.

1.3 HOMOLOGOUS RECOMBINATION IN THE rDNA ARRAY

Repression of recombination is an important function of rDNA silent chromatin because repetitive elements are prone to recombination and unequal sister chromatid exchange. Recombination at the rDNA can occur between rDNA repeats on the same strand of chromatin, between repeats on sister chromatids, or between repeats on homologous chromosomes; models discussed here will focus on mitotic homologous recombination in haploid cells. Destabilization of the rDNA locus caused by high levels of recombination results in the formation of extrachromosomal rDNA circles (ERCs), shortened replicative lifespan (how many times a mother cell can divide), and expansion or reduction in the number of rDNA repeat units [62, 63]. The NTS1 region of an rDNA repeat unit is critical for rDNA recombination as it is the site of the replication fork

barrier, where DNA double stranded breaks can induce recombination as a repair mechanism.

Cohesin is a protein complex that bonds sister chromatids together following replication in S-phase until separation in anaphase of mitosis. Four subunits of cohesin are Smc1, Smc3, Scc3 and Mcd1 (Scc1). Different silencing proteins have been shown to interact with cohesin subunits to repress homologous recombination in the rDNA array. The cohibin protein Csm1 may interact with cohesin subunits Mcd1 and Smc1, which supports a mechanism by which cohibin represses recombination and unequal sister chromatid exchange by stabilizing the sister chromatid interaction with cohesin and tethering them to the inner nuclear membrane to restrict chromatid movement [57, 64, 65].

In cells lacking Sir2, the rate of unequal sister chromatid exchange is increased and the association of the Mcd1 subunit of cohesin is reduced compared to *SIR2*⁺ cells. The reduction in Mcd1 association is indicative of reduced interaction of the rDNA with cohesin, which was proposed to be the cause of increased recombination in *sir2Δ* cells [66]. In addition, Sir2 represses Pol II transcription from E-pro in NTS1; Pol II transcription of E-pro causes dissociation of cohesin, which leaves the region more prone to recombination and is further evidence that the Sir2-cohesin interaction is important for repressing recombination in the rDNA [24]. These two interactions of cohesin with the rDNA, through Csm1 and Sir2, are likely independent. Data show that loss of Sir2 in cells lacking Csm1 (and other CLIP and cohibin proteins) results in an

additive increase in the rate of recombination, suggesting that Sir2 represses recombination independently of the tethering proteins [56, 57].

The protein Fob1 is another important element of homologous recombination at the rDNA. Fob1 binds the rDNA at the replication fork barrier in NTS1 and is responsible for recruiting the RENT complex (therefore Sir2) and Tof2 (leading to cohibin and CLIP interactions) to the rDNA [22, 57]. Sir2, cohibin and CLIP all have a role in repressing recombination, and the loss of Fob1 decreases the association of these proteins with the rDNA. However, the loss of Fob1 does not result in an increase in recombination at the rDNA array; in fact, Fob1 is required for recombination, indicating a dual role for Fob1 in both repressing and promoting rDNA recombination [21, 63].

While repression of recombination is important for genome stability, controlled homologous recombination is an important mechanism for a cell to regulate the number of rDNA repeats in the rDNA array. Homologous recombination between rDNA repeats allows cells to remove extra copies of the rDNA repeat units, replace rDNA repeats that are lost during DNA replication or ERC formation, or to remove damaged rDNA repeats [25, 40, 63]. Fob1 is required for these processes, and recent experiments are beginning to unveil new details about how Fob1 promotes recombination.

When regions of DNA are brought in close proximity to each other through protein interactions, it is called “chromosome kissing”. Chromosome kissing can control many regulatory activities, and Fob1 has recently been shown to promote this interaction at the rDNA [67]. Fob1 binding at the replication fork barrier occurs at two DNA sequences called Ter1 and Ter2 [68]. Fob1 can bind to itself, or oligomerize [68];

oligomerization of Fob1 is dependent on phosphorylation of the C-terminal end of Fob1 to allow interactions between the N-terminal ends of Fob1 proteins [67]. Fob1 mutants that are unable to oligomerize (due to loss of phosphorylation sites in the C-terminus) have reduced recombination between chromosomal rDNA and plasmid-borne rDNA, whereas Fob1 mutants that lack the C-terminus, and therefore may always oligomerize, have increased recombination. Interestingly, Fob1 oligomerization does not seem to be required for recombination between sites on the same chromosome [67]. While this study provides insight into the promotion of recombination by Fob1, there are still unanswered questions about this role of Fob1.

1.4 OTHER SILENT DOMAINS IN *S. cerevisiae*

Silent chromatin is known to exist in *S. cerevisiae* in two other domains of the genome besides the rDNA: the homothallic mating type loci, *HML* and *HMR*, and the telomeres [6, 69-71]. The *HM* loci were the first silent chromatin domains discovered in *S. cerevisiae*, followed by the telomeres and rDNA [26, 28, 72-74]. The mechanisms that regulate silent chromatin at the *HM* loci and telomeres have been characterized more fully than those that act at the rDNA [6]. Silent chromatin at the *HM* loci and telomeres is discussed in more detail below.

1.4.1 Silent chromatin at the *HM* loci

In addition to the *MAT* locus, which determines if a cell is mating type \underline{a} or α , each *S. cerevisiae* cell harbors silenced copies of the \underline{a} and α genes at the homothallic mating (*HM*) type loci, *HMR* and *HML*, respectively. Silent chromatin at the *HM* loci is

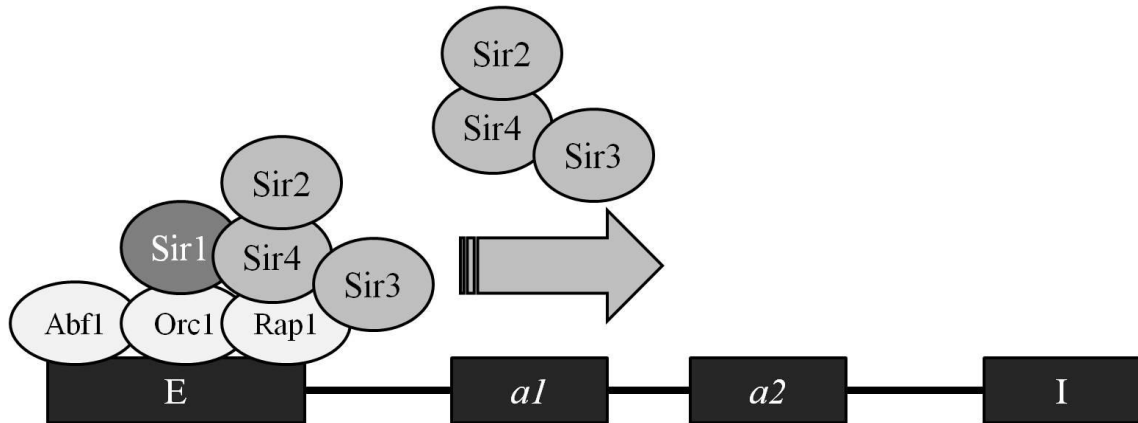
required for haploid cells to maintain a haploid mating type. The establishment of silent chromatin at the *HM* loci has been studied in detail; the silent chromatin established at the *E* silencer of the *HMR* locus is described here as an example (Figure 1-5A); requirements for the establishment of silent chromatin and the SIR proteins at the *HML* silencers are similar to that described here for the *HMR-E* silencer [75].

The *HMR* locus contains the *a1* and *a2* genes flanked by two silencers, *E* and *I*, that are responsible for recruiting several proteins. The *I* silencer is not functional without the *E* silencer, and it is proposed that the SIR silencing complex assembles at the *E* silencer and spreads to the *I* silencer [75]. The *E* silencer contains binding sites for several proteins, including Abf1, Rap1, and ORC (Origin Recognition Complex), which recruit the SIR proteins. Sir1 interacts with Orc1 to begin the assembly of SIR proteins at the silencer [76, 77]. Rap1 interacts with Sir3 and Sir4 [78, 79]. Sir4 also interacts with Sir1, and recruits Sir2 and Sir3 [6, 75, 80, 81]. Once these silencing proteins have assembled at the *E* silencer, Sir2 and Sir3 promote the spreading of silent chromatin to the *a1* and *a2* genes in *HMR*. Sir2's deacetylase activity, which is enhanced by Sir4, is necessary for spreading; Sir3 interacts with histone H4 tails that have been deacetylated at lysine sixteen by Sir2 [32, 33, 75, 82-85].

1.4.2 Silent chromatin at the telomeres

Telomeres are maintained in a heterochromatic state to protect the ends of chromosomes from shortening due to replication and recombination. Telomere position effect, a form of Pol II gene silencing, was documented in the early 1990s, when it was found that expression of a Pol II reporter gene integrated near the end of a chromosome

A.



B.

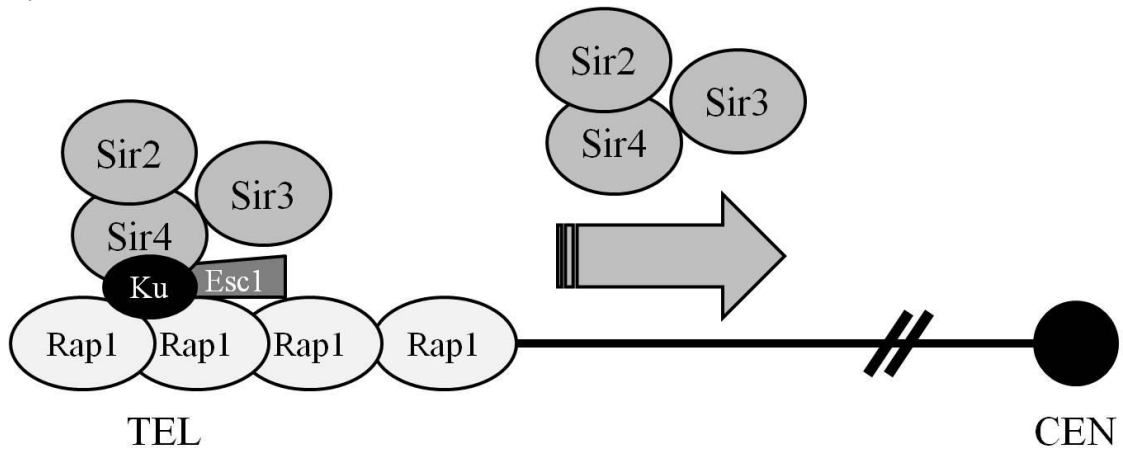


Figure 1-5. Silent chromatin at the *HM* loci and telomeres. (A) Silent chromatin at the *HM* loci requires binding of SIR proteins to the *E* silencer. The *E* silencer contains binding sites for the proteins Abf1, Orc1 and Rap1. Sir1 binds to Orc1; recruitment of Sir2, Sir3 and Sir4 follows. The Sir2-Sir3-Sir4 complex spreads silent chromatin through the *HMR* locus. *E*, *E* silencer; *I*, *I* silencer. *a1* and *a2*, genes required for expression of an α mating type. (B) Silent chromatin at the telomeres requires Rap1, Ku70/Ku80 (Ku), and Esc1 to recruit Sir4. Sir4 interacts with Sir2 and Sir3; the Sir2-Sir3-Sir4 complex spreads silent chromatin into the chromosome toward the centromere. TEL, telomere; CEN, centromere. Large arrow, direction of spread of silent chromatin.

was lower than expression of reporter genes located farther away from the telomere [74]. Similar to the *HM* loci, silent chromatin at the telomeres requires Rap1, Sir2, Sir3, and Sir4 [86]. In contrast, Sir1 is not required for silent chromatin at the telomeres. At telomeres, multiple Rap1 proteins bound to the DNA recruit Sir3 and Sir4 (Figure 1-5B) [87]. In addition to Rap1, Sir4 interacts with the telomeric protein heterodimer Ku70/Ku80 and Esc1 [70, 88, 89]. Once Sir4 is bound to the telomeres, Sir2 and Sir3 are recruited and silent chromatin spreads into the chromosome in a manner similar to spreading of the Sir2-Sir3-Sir4 complex at the *HMR* locus [6, 70].

1.4.3 Shared silencing mechanisms

Sir2 is shared by all three silent chromatin domains in *S. cerevisiae*. The discovery of Sir2 in silencing the *HM* loci provided researchers guidance to identifying silencing proteins at the telomeres and rDNA when it was discovered that these two domains are heterochromatic. Although Sir2 is required for silencing at all three silenced regions, the proteins Sir2 associates with vary. Association of Sir2, Sir3 and Sir4 is important for the formation of silent chromatin at the *HM* loci and telomeres, whereas Sir2 association with Net1 and Cdc14 to form the RENT complex is necessary for the formation of silent chromatin at the rDNA. Sir2 is limiting for rDNA silent chromatin, and Sir2 will preferentially bind to the *HM* loci and telomeres when Sir2 is reduced in the cell [36]. Thus, silent chromatin at the *HM* loci and telomeres modulates rDNA silent chromatin.

The location of the *HM* loci, telomeres, and rDNA in the nucleus is similar; all are found close to the nuclear membrane. Tethering of the rDNA to the inner nuclear

membrane was discussed in Section 1.2.4, and requires interaction of CLIP, cohibin, Tof2, and Fob1. Loss of tethering results in altered silent chromatin at the rDNA. Telomeres are also tethered to the inner nuclear membrane through interactions that involve cohibin, CLIP, Sir3, Sir4, Esc1 and Ku [70, 90-92]. CLIP and cohibin proteins are required for tethering of both the telomeres and the rDNA array. Studies of the spatial location of chromosomes in the nucleus and tethering of chromosomes to the inner nuclear membrane are being done using the telomeres and the rDNA array to study how spatial organization in the nucleus affects homologous recombination [61, 93]. This is the most recent example of how the understanding of one silenced locus can aid in the understanding of another silent locus.

1.5 FROM YEAST TO MAMMALS

S. cerevisiae is a relatively simple model for investigating the role of modified histones and other protein interactions in the establishment of heterochromatin because there is no RNAi system and there is no DNA methylation [14, 15]. Because there are fewer proteins with redundant functions in *S. cerevisiae* than in mammalian cells, *S. cerevisiae* is a tractable system for performing mutational analysis to determine the function of specific residues within proteins.

There are many histone marks in *S. cerevisiae* that are conserved hallmarks of heterochromatin, for example, deacetylated H3K9 and H3K14. The study of modified histones in *S. cerevisiae* has provided fundamental information necessary to elucidate the function of additional histone modifications and DNA modifications in higher

eukaryotes. In addition, the study of histone crosstalk, which is how modifications on histone tails influence other modifications, was discovered and is often pursued in yeast. The first direct evidence for histone crosstalk was that phosphorylation of Serine 10 in the histone H3 tail promotes acetylation of H3K14 in *S. cerevisiae* [94]. In 2002 it was found in *S. cerevisiae* that ubiquitination of H2BK123 is necessary for methylation of H3K4; this interplay between H2BK123 ubiquitination and H3K4 methylation is conserved in mammalian cells [95-98].

Sir2 is one of the most widely studied silencing proteins. It was discovered in yeast for its role in silencing of the *HM* loci, and has homologs in higher eukaryotes; the human homolog of Sir2 is SIRT1, one of seven SIR proteins in mammals. Similar to the requirement of yeast Sir2 in silent chromatin, SIRT1 is key for heterochromatin in mammals and regulates the rDNA. In mammals, SIRT1-7 have been implicated in numerous pathways and regulatory roles, including cell differentiation, muscle development and many cancers [34, 99-102]. Study of Sir2 in *S. cerevisiae* has established the role of Sir2 in heterochromatin as an NAD-dependent histone deacetylase; these studies are the foundation for determining the function and role of the mammalian SIRT1-7. There is still much to learn about the action of Sir2 in heterochromatin establishment and maintenance in yeast. New information about Sir2 regulation and silent chromatin is being discovered; for example, it was recently shown that sumoylation of Sir2 can affect its ability to bind Sir4, but that sumoylated Sir2 still localizes to the nucleolus. This may be a form of regulation to direct Sir2 to the rDNA instead of to the *HM* loci or telomeres where it interacts with Sir4 to establish silent

chromatin [103]. New information such as this is likely to lead to a better understanding of SIRT1-7 in mammalian systems, just as the discovery of Sir2 in *S. cerevisiae* led to the discovery of SIRT1-7 in mammals.

The location of heterochromatin at the nuclear periphery is common between yeast and mammals [104]. Tethering of the telomeres to the nuclear periphery is critical for telomere maintenance in *S. cerevisiae* and mammalian cells; loss of telomere length is associated with aging and diseased states [105]. Misregulation of the rDNA in yeast causes increased recombination, leading to genome instability and shortened replicative life span, while misregulation of the rDNA is found in many diseased mammalian cells, including cancer and neurodegenerative disorders [106, 107]. For example, in approximately half of cells examined from lung and colorectal cancer patients the size of the rDNA fragments in tumor cells was different from that in non-tumor cells from the same patient [108]. Change in the rDNA is also associated with neurodegenerative disease; rDNA content was measured by quantitative PCR in brain cells and was found to be increased in cells from patients diagnosed with dementia with Lewy bodies compared to age-matched samples of patients with no dementia [13]. Knowledge of *S. cerevisiae* heterochromatin and rDNA regulation provides guidance for studies investigating the relationship between diseased states and misregulated rDNA in mammals.

1.6 DISSERTATION OVERVIEW

Research presented in this dissertation was focused on improving understanding of silent chromatin in individual rDNA repeats in *S. cerevisiae*. First, a Sir2 Tet-Off system was developed and characterized to study how Sir2 regulates silent chromatin in individual rDNA repeat units. The ability to turn *SIR2* off for a few hours (before turning it back on) allows silent chromatin at the rDNA to be disrupted without permitting high levels of recombination. In the future, this system will be used to study the establishment of silent chromatin at the rDNA and other loci.

Silent chromatin at the rDNA array has been assumed to be uniform across the ~1.8 Mb locus. By evaluating two processes repressed by silent chromatin at the rDNA, Pol II transcription and mitotic recombination, it was discovered that silent chromatin is not the same in different rDNA repeat units. Chromatin immunoprecipitation experiments revealed that association of Sir2 and modified histones varies with the strength of silent chromatin in individual rDNA repeats; more silent rDNA repeats are associated with more Sir2. These findings suggest that silent chromatin at the rDNA is not static; instead, individual rDNA repeats, that share the same primary DNA sequence, acquire chromatin structures that are more dynamic than previously appreciated.

Lastly, in experiments originally designed to screen for mutants of the histone methyltransferase Set1 that affected rDNA silent chromatin, a new phenotype of *set1Δ* cells was identified. Cells that lack the histone methyltransferase activity of Set1 were found to be sensitive to the herbicide 3-Amino-1,2,4-triazole, a competitive inhibitor of the *HIS3* gene product. Experiments demonstrated that this phenotype is not specific to

Set1 function at the rDNA; whether the *HIS3* gene is inside or outside of the rDNA, cells lacking H3K4 methylation are sensitive to 3-AT. When grown in the presence of 3-AT, cells that lack of H3K4 methylation express *HIS3* at lower levels than wild-type cells. This study identified regulation of *HIS3* gene expression by Set1. These findings reveal a functional relationship between the histone methyltransferase activity of Set1 and gene regulation outside of the rDNA.

Additional information about silent chromatin at the rDNA, how it is established and regulated by silencing proteins and modified histones, is crucial for better understanding of the role of the rDNA in cell viability. As knowledge of the rDNA chromatin increases, the importance of proper regulation of the rDNA repeats becomes more apparent, as evidenced by the growing list of phenotypes and diseases associated with improper regulation of the rDNA. Moreover, the rDNA is an invaluable tool for studying the role of modified histones in heterochromatin function, a process that is conserved throughout eukaryotes.

II. A TETRACYCLINE-REGULATABLE SYSTEM TO CONTROL SIR2 EXPRESSION AND STUDY THE ESTABLISHMENT OF SILENT CHROMATIN AT THE RIBOSOMAL DNA LOCUS IN *Saccharomyces cerevisiae*

2.1 INTRODUCTION

A system derived from bacterial repression systems to regulate transcription of RNA Polymerase (Pol) II transcribed genes in eukaryotes was developed in the early 1990s [109-111]. The Tet-Off regulatory system is based on the *Escherichia coli* Tn10 tetracycline repressor (TetR) and it has been modified for use in yeast to control gene expression and to study essential genes [112-116]. There are two components of the Tet-Off system; the promoter of a gene of interest is replaced with the bacterial *tet* operator element (*tetO*), often either two (*tetO*₂) or seven (*tetO*₇) copies of the *tet* operator sequence, and the *S. cerevisiae* *CYC1*_{TATA} element. Transcription from the *tetO*_N-*CYC1*_{TATA} promoter is regulated by a tetracycline-repressible transactivator fusion protein (tTA) that is expressed from a constitutive promoter. The tTA is a heterologous protein containing a Pol II-transcriptional activator fused to the *E. coli* Tn10 Tet repressor DNA binding domain (TetR_{DBD}) (Figure 2-1). The tTA fusion protein binds the *tetO* sequences, allowing the activator to promote transcription of the gene of interest (Figure 2-2A). When doxycycline, a stable analog of tetracycline [111, 112, 117], is added to growth media, the tTA binds preferentially to doxycycline instead of the *tetO* sequences, removing the tTA from the promoter region and thereby halting activation of

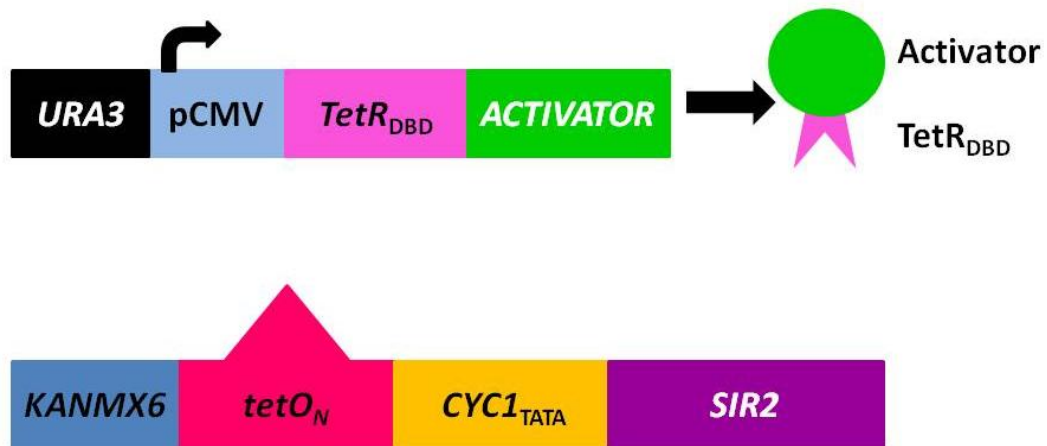


Figure 2-1. Components of the Tet-Off system are integrated into yeast cells to regulate expression of the *SIR2* gene. Top, cassette encoding the tetracycline-repressible transcriptional transactivator fusion protein (referred to as tTA). Transcription of the cassette is driven by the Cytomegalovirus early promoter (pCMV). The sequences encoding the Tet repressor DNA binding domain (TetR_{DBD}) and transcriptional activator are transcribed together producing the tTA fusion protein; the *URA3* gene is used for selection of yeast transformants. Bottom, the endogenous *SIR2* promoter is replaced with two or seven copies of the *tet* operator (*tetO_N*) and the TATA element from *CYC1* (*CYC1_{TATA}*). The *KANMX6* sequence allows for selection of G418-resistant yeast transformants.

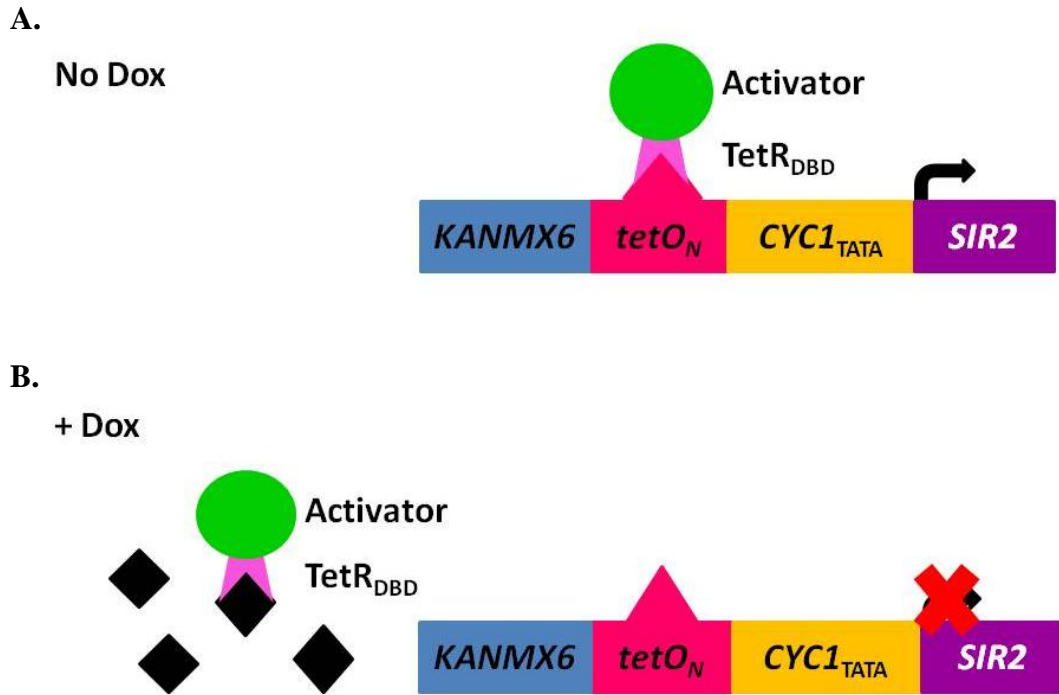


Figure 2-2. Tet-Off system controls expression of *SIR2*. (A) When cells are grown in media without doxycycline (Dox) the TetR_{DBD} of the tTA binds to the *tet* operator (*tetO_N*), bringing the transcriptional activator in close proximity of *SIR2*. (B) Upon the addition of doxycycline to growth media, the TetR_{DBD} binds doxycycline preferentially over the *tetO_N*, removing the activator from the promoter, effectively stopping *SIR2* transcription.

transcription of the gene of interest (Figure 2-2B). When doxycycline is removed or no longer present in the cells, the tTA will re-bind the *tetO* sequences and drive transcription of the gene of interest.

The level of gene expression induced by a Tet-Off system can be changed by altering the number of *tetO* sequences (*tetO*₂ or *tetO*₇) in front of the gene of interest; more operators provide more tTA binding sites, thereby increasing the association of the activator and allowing for a higher level of transcription [112]. In addition, the strength of the transcriptional activator in the tTA influences the level of transcription of the gene of interest. The Virion Protein 16 (VP16) from Herpes Simplex Virus is an activator that induces transcription to high levels [118] and is used commonly in Tet-Off systems. A weaker activator is the activation domain of Gcn4 (Gcn4A); native to *S. cerevisiae*, endogenous Gcn4 induces many genes in response to amino acid starvation and stress [119-121]. Fusion of the transcriptional activator domain to the TetR_{DBD} ensures that the activator is recruited only to the gene(s) driven by the exogenous *tetO*_N-*CYC1*_{TATA} promoter.

Silent information regulator 2 (Sir2) protein is an NAD⁺-dependent histone deacetylase required for silent chromatin in the ribosomal DNA (rDNA) locus and other loci in *S. cerevisiae*. Deletion of the *SIR2* gene from yeast cells causes the loss of silent chromatin at the rDNA, leading to higher levels of Pol II transcription and homologous recombination within the rDNA [18, 26-30, 32]. In *sir2Δ* cells, homologous recombination between rDNA repeat units changes the length of the rDNA locus and generates extra-chromosomal rDNA circles [40, 62, 122]. The destabilization of the

rDNA in cells lacking *SIR2* makes it difficult to study mechanisms that regulate silent chromatin in the rDNA.

To study the specific mechanisms of Sir2 in rDNA silent chromatin without destabilizing the rDNA locus, a system has been developed to regulate the expression of *SIR2*. Expression of the endogenous *SIR2* gene is placed under the control of a tetracycline repressible promoter. The *SIR2* Tet-Off system is expected to provide quick and reversible repression of the *SIR2* gene. The goal is to establish an experimental system in which the addition of doxycycline to growth media causes the depletion of Sir2 protein from cells, and after removal of doxycycline from the media, allows the re-expression of the *SIR2* gene. The system will be used to investigate how Sir2 regulates silent chromatin at the rDNA. For example, the system will be used to follow the dissolution and re-establishment of silent chromatin at the rDNA as Sir2 protein levels decrease and increase, respectively, in the cell. The shutdown phase when the level of Sir2 is decreasing/absent will be as brief as possible in order to minimize rearrangement of the rDNA array.

A variety of Tet-Off systems to regulate *SIR2* expression are presented here, as are experiments to analyze the function of rDNA silent chromatin when using the Tet-Off system to regulate *SIR2* expression. The *SIR2* Tet-Off system will be optimized for future studies to study the establishment of rDNA silent chromatin and lead to a better understanding of chromatin domains in repeated DNA sequences.

2.2 MATERIALS AND METHODS

2.2.1 Media

Growth media used in these experiments has been described previously [123]. YPADT is YPD media supplemented with 40 mg/L adenine hemisulfate and 80 mg/L L-tryptophan. YPADTU is YPADT media supplemented with 20 mg/L uracil. When indicated, doxycycline was added to media immediately prior to use at a concentration of 10 µg/ml.

2.2.2 Yeast strains used in this study

All *S. cerevisiae* strains and plasmids used in this study are listed in Table 2-1. Yeast strains were transformed by lithium acetate transformation [124] with the following modifications. Saturated cultures grown in YPADT or YPADTU were diluted into fresh YPADT or YPADTU at $\sim 4 \times 10^6$ cells/ml and grown to $1-2 \times 10^7$ cells/ml. Cells were harvested by centrifugation and washed with 10 ml sterile water, then resuspended in 1 ml 1X TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA, pH 8.0)/0.1 M Lithium acetate (LiAc) and pelleted for 2 minutes at 5K rpm. Cells were resuspended in 100 µl 1X TE/0.1 M LiAc per 20 ml original culture. 100 µl aliquots were incubated with 50 µg carrier DNA and 1-10 µg transforming DNA at 30°C for 30 minutes, followed by incubation at 30°C for 30 minutes with 40% Polyethylene glycol 3350/1X TE/0.1 M LiAc. Cells were heat shocked at 42°C for 15 minutes, washed with sterile water, plated on selective media and incubated at 30°C until colonies appeared. For transformations using a *KANMX* marker, cells were incubated for two hours in rich media at 30°C on a roller drum prior to plating on selective media.

Table 2-1. Yeast strains and plasmids used in this study.

Name	Genotype	Activator	<i>tetO</i>
MBY2508	<i>MAT_a (his3Δ200) leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0 NTS2::HIS3</i>		
MBY2510	<i>MAT_a (his3Δ200) leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0 NTS2::HIS3</i>		
MBY2570	<i>MAT_a (his3Δ200) leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0 chr XII::HIS3</i>		
MBY2680	MBY2508 <i>sir2Δ::LEU2</i>		
MBY2687	MBY2510 <i>sir2Δ::LEU2</i>		
MBY2752	<i>MAT_a his3-1 leu2Δ0 met15Δ0 URA3::CMV-tTA (Timothy Hughes, R1158)</i>	VP16	
MBY2776	MBY2508 <i>URA3::CMV-VP16-tTA</i>	VP16	
MBY2782	MBY2776 <i>KANMX6-tetO₇-CYC1_{TATA}::SIR2</i>	VP16	<i>tetO₇</i>
MBY2831	MBY2570 chr I::pUI-tTA-GCN4A-ADH1 _{term} -URA3 <i>KANMX6-tetO₇-CYC1_{TATA}::SIR2</i>	Gcn4A	<i>tetO₇</i>
MBY2849	MBY2776 <i>KANMX6-tetO₂-CYC1_{TATA}::SIR2</i>	VP16	<i>tetO₂</i>
MBY2850	MBY2776 <i>KANMX6-tetO₂-CYC1_{TATA}::SIR2</i>	VP16	<i>tetO₂</i>
MBY2851	<i>MAT_a (his3Δ200) leu2Δ0 met15Δ0 trp1Δ63 (ura3Δ0) NTS2::HIS3 chr I::pUI-tTA-GCN4A-ADH1_{term}-URA3 KANMX6-tetO₂-CYC1_{TATA}::SIR2</i>	Gcn4A	<i>tetO₂</i>
MBY2852	MBY2570 chr I::pUI-tTA-GCN4A-ADH1 _{term} -URA3 <i>KANMX6-tetO₇-CYC1_{TATA}::SIR2</i>	Gcn4A	<i>tetO₇</i>
MBY2884	MBY2508 chr I::pUI-tTA-GCN4A-ADH1 _{term} -URA3 <i>KANMX6-tetO₇-CYC1_{TATA}::SIR2</i>	Gcn4A	<i>tetO₇</i>
MBY2885	MBY2570 chr I::pUI-tTA-GCN4A-ADH1 _{term} -URA3 <i>KANMX6-tetO₇-CYC1_{TATA}::SIR2</i>	Gcn4A	<i>tetO₇</i>
MBY2933	MBY2510 chr I::pUI-tTA-GCN4A-ADH1 _{term} -URA3	Gcn4A	
MBY2952	MBY2933 <i>KANMX6-tetO₂-CYC1_{TATA}::SIR2</i>	Gcn4A	<i>tetO₂</i>
Plasmid	Description		
MBB689	pKan- <i>tetO₇</i> (Timothy Hughes, RP188) [114]		
MBB690	pKan <i>tetO₂</i> [125]		
MBB692	pUI-tTA-GCN4A-ADH1 _{term} -URA3 [125]		

Table 2-2. Oligonucleotides used in this study.

Name	Sequence
OM362	CATCTCATGGATCTGCACATG
OM363	CTGTTACTTGGTTCTGGCGAG
OM1092	GATGTAAAGCCCATTCTCACGTATTTCAAGAAATTAGGCATCG CAGCGGATAACAATTTACACACAGGA
OM1093	GCTAGTCTTTGATACGGCGTATTTTCATATGTGGGATGGTCATG GATCCCCCGAATTGATC
OM1106	GTAAAGCCCATTCTCACGTATTTCAAGAAATTAGGCATCGCCA GCTGAAGCTTCGTACGC
OM1107	GCTAGTCTTTGATACGGCGTATTTTCATATGTGGGATGGTATAG GCCACTAGTGGATCTG

Yeast strain R1158 (MBY2752), containing the VP16-tTA and plasmid RP188 (MBB689) containing the *KANMX6-tetO₇-CYC1_{TATA}* cassette were gifts from Timothy Hughes and have been described previously [114, 126]. VP16-tTA was amplified from genomic DNA of MBY2752 with primers specific to the *URA3* locus (OM362 and OM363, Table 2-2). Resulting PCR product was used in an integrative transformation to integrate the VP16-tTA into the *ura3Δ0* locus. *KANMX6-tetO₇-CYC1_{TATA}* was amplified from plasmid MBB689 with oligonucleotides OM1092 and OM1093 (Table 2-2); resulting PCR product contained sequences homologous to the *SIR2* promoter to allow for replacement of the *SIR2* promoter with the *KANMX6-tetO₇-CYC1_{TATA}* cassette via homologous recombination.

Plasmids containing Gcn4A-tTA (MBB692) and *KANMX6-tetO₂-CYC1_{TATA}* (MBB690) cassettes were gifts from Daniel Gottschling and have been described previously [125]. Gcn4A-tTA was amplified from MBB692 with primers adding homologous sequences for integration of the cassette into an intergenic region of chromosome *I* as previously described [125]. *KANMX6-tetO₂-CYC1_{TATA}* was amplified with primers OM1106 and OM1107 (Table 2-2) to add sequence homology to the *SIR2* promoter to allow for replacement by integrative lithium acetate transformation via homologous recombination.

2.2.3 CHEF gel analysis and mapping *HIS3* in the rDNA array

Experiments were performed as previously described [26, 127] with the following modifications. Chromosomal DNA embedded in low melt agarose plugs was prepared from saturated cultures or from aliquots of cultures during a time course, grown

in YPADT or YPADTU medium, with or without 10 µg/ml doxycycline. Plugs were incubated with 1 mM phenylmethylsulfonyl fluoride (PMSF) and washed extensively prior to restriction digest with 40-80 units *Pst*I (New England Biolabs) at 37°C overnight. Undigested plugs incubated without restriction enzyme were analyzed in some experiments. Chromosomal DNA and a lambda PFG ladder (New England Biolabs) were separated on a 1% agarose gel in 0.5X TBE buffer at 14°C by electrophoresis in a BioRad CHEF mapper, using 6 V/cm 60 second pulses for 15 hours followed by 6 V/cm 90 second pulses for 9 hours at an angle of 120°. The gel was stained with ethidium bromide and imaged. After 2 minutes of exposure to UV light in a Stratalinker 2400 (Stratagene) to nick the DNA fragments, gels were incubated shaking at room temperature for 30 minutes each in denaturation solution (0.2 N NaOH, 0.6 M NaCl), followed by neutralization solution (1.5 M NaCl, 1 M Tris, pH 7.4) and 10X SSC (1.5 M NaCl, 0.15 M HOC(COONa)(CH₂COONa)•2H₂O, pH 7.0-7.5). DNA was transferred to Genescreen, Hybond, or Hybond-XL membrane (Amersham) by blotting with 10X SSC for 12-16 hours.

Membranes were first hybridized to ³²P-labeled DNA probes made by random priming using the *HIS3* gene as a template. Blots were imaged using a Pharos FX Plus Molecular Imager (BioRad, Hercules, CA). After imaging, the membrane was stripped for two hours at 70°C with 50% formamide in 1X SSC (0.15 M NaCl, 0.015 M HOC(COONa)(CH₂COONa)•2H₂O, pH 7.0-7.5). The membrane was then incubated with a ³²P-labeled DNA probe made by random priming using the rDNA NTS as a template. After imaging, the sizes of labeled rDNA fragments were estimated from the

lambda PFG ladder, which were then used to determine the location of *HIS3* gene within the rDNA array and the length of the rDNA array.

2.2.4 Whole cell extract and Western blot analysis of Sir2

Whole cell protein extracts were prepared as described previously with the following modifications [128]. In some experiments, 20-50 ml cells were harvested; if 20-25 ml cells were harvested only 0.125 ml RIPA buffer was used. Protein (80 µg) from whole cell extracts was separated on 10% SDS-PAGE, transferred to PVDF membrane and probed with α -Sir2 yN-19 (sc-6666, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:1000) primary antibody. Antibody binding was detected with IgG-HRP-conjugated secondary antibody donkey α -goat (sc-2020, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:15,000) and Clarity Western ECL Substrate (Bio-Rad, Hercules, CA). As a loading control the membrane was probed with α -phosphoglycerate kinase (Pgk1) primary antibody (459250, Invitrogen, Camarillo, CA; 1:2000) and detected with IgG-HRP conjugated α -mouse secondary antibody (W402B, Promega, Madison, WI; 1:15,000) and Clarity Western ECL Substrate. Blots were imaged with Bio-Rad Molecular Imager ChemiDoc XRS and quantified with Quantity One software.

2.2.5 Northern blot analysis of *HIS3*

Cells were harvested for RNA extraction as previously described [129] with the following modifications. Cells were resuspended in 450 µl AE buffer (50 mM $C_2H_3NaO_2$ pH 5.3, 10 mM EDTA) transferred to a microfuge tube, and 50 µl 10% SDS was added. Extraction with an equal volume of chloroform:isoamyl alcohol (24:1) was

performed before the addition of 50 μ l 3M sodium acetate pH 5.3 and 2.5 volumes 100% ethanol. After precipitation, RNA was resuspended in 20-60 μ l sterile water and stored at -70°C. RNA (20 μ g) was analyzed by Northern blot as previously described [130]. ³²P-labelled DNA probes synthesized by random priming were hybridized to membranes to measure the steady state level of *HIS3* transcript. A ³²P-labelled DNA probe specific for *ACT1* was used as a loading control. Blots were imaged using a Pharos FX Plus Molecular Imager (BioRad, Hercules, CA) and quantified with Quantity One software.

2.2.6 Doxycycline time courses

Saturated cultures were used to inoculate duplicate cultures at $\sim 4 \times 10^6$ cells/ml in YPADTU. Doxycycline was added from a 5 mg/ml stock solution in sterile water to one of the duplicate cultures at a concentration of 10 μ g/ml. Cultures were grown shaking at ~ 250 -300 rpm on a platform shaker at 30°C. Aliquots of 20-50 ml of culture were taken at indicated time points and cells were harvested for whole cell protein extract (described in Section 2.2.4) or RNA extraction (described in Section 2.2.5). One to ten ml of culture was used for agarose-embedded chromosome plugs (described in Section 2.2.3). To remove doxycycline from cultures, cells were filtered onto sterile filter paper by vacuum filtration and washed with approximately ten-fold volume of sterile water. The cells on the filter paper were inoculated into fresh YPADTU at $\sim 4 \times 10^6$ cells/ml and incubated at 30°C on a platform shaker set at ~ 250 -300 rpm.

2.3 RESULTS

2.3.1 Cells lacking Sir2 have hyper-recombination and movement of the *HIS3*-marked rDNA repeat within the rDNA array

Integration of a Pol II transcribed gene, such as the *S. cerevisiae HIS3* gene, is a common method used to study silent chromatin at the rDNA. Both Pol II transcription and mitotic recombination in the rDNA are repressed by silent chromatin at the rDNA locus; changes in Pol II transcriptional silencing of *HIS3* can be assessed by Northern blot and mitotic recombination rates can be assessed by measuring the loss of the *HIS3* gene during growth on non-selective medium [26-30]. The histone deacetylase activity of Sir2 is required for silent chromatin at the rDNA [30]. When Sir2 is absent from the cell, transcription of a *HIS3* reporter gene located in the rDNA is increased and the rDNA repeats participate in recombination at high levels. Recombination between rDNA repeats destabilizes the rDNA, making it difficult to study silent chromatin because the *HIS3*-marked rDNA repeat can be lost or moved during recombination. To assess the extent of movement of a *HIS3* reporter gene in the rDNA in the absence of Sir2, *SIR2* was deleted in strains that each contain a single *HIS3* gene in a single rDNA repeat unit.

Whole chromosomes from five colonies of the same *sir2Δ* strain (MBY2680) were digested with the restriction enzyme *PstI* and separated on a CHEF gel. The *HIS3* gene introduces a single *PstI* restriction site into the rDNA array, therefore digestion with *PstI* cuts the rDNA into two fragments; the size of the fragments are determined by the location of the *HIS3*-marked rDNA repeat unit within the rDNA array. Southern blot

analysis with a ^{32}P -labeled probe complementary to the 5' end of the *HIS3* gene revealed the size of the *HIS3*-containing fragment; a change in fragment size among the colonies indicates movement of the *HIS3*-marked rDNA repeat within the rDNA array.

Movement of the *HIS3*-marked rDNA repeat was detected in each of the five *sir2* Δ colonies, compared to each other and compared to the *SIR2* $^{+}$ parent strain (MBY2508) (Figure 2-3). The size difference between the largest and smallest *HIS3* fragment in *sir2* Δ colonies is approximately 17 rDNA repeats. The *SIR2* $^{+}$ parent, MBY2508, has been mapped in three independent experiments; the size of the *HIS3*-fragment of MBY2508 mapped to the same location within less than two rDNA repeats (data not shown). Chromosomal fragments from *sir2* Δ cells are blurry and have higher background signal compared to fragments from the *SIR2* $^{+}$ strain; this is due to the instability of the rDNA in *sir2* Δ cells and the resulting change in the size of the rDNA caused by hyper-recombination. Movement of the *HIS3*-marked rDNA repeat was observed in colonies of one other *sir2* Δ strain (MBY2687) with a single *HIS3* integrated into the rDNA array; the difference in size between the largest and smallest *HIS3* fragments from five *sir2* Δ colonies was approximately 35 rDNA repeats (data not shown). MBY2510, the *SIR2* $^{+}$ parent of MBY2687, has been mapped in three independent experiments, and the *HIS3*-fragment mapped to the same location in the rDNA array within five rDNA repeats. The movement of the *HIS3*-marked rDNA repeat and destabilization of the rDNA in *sir2* Δ cells hinder mechanistic studies to determine the role of Sir2 in the regulation of silent chromatin at the rDNA.

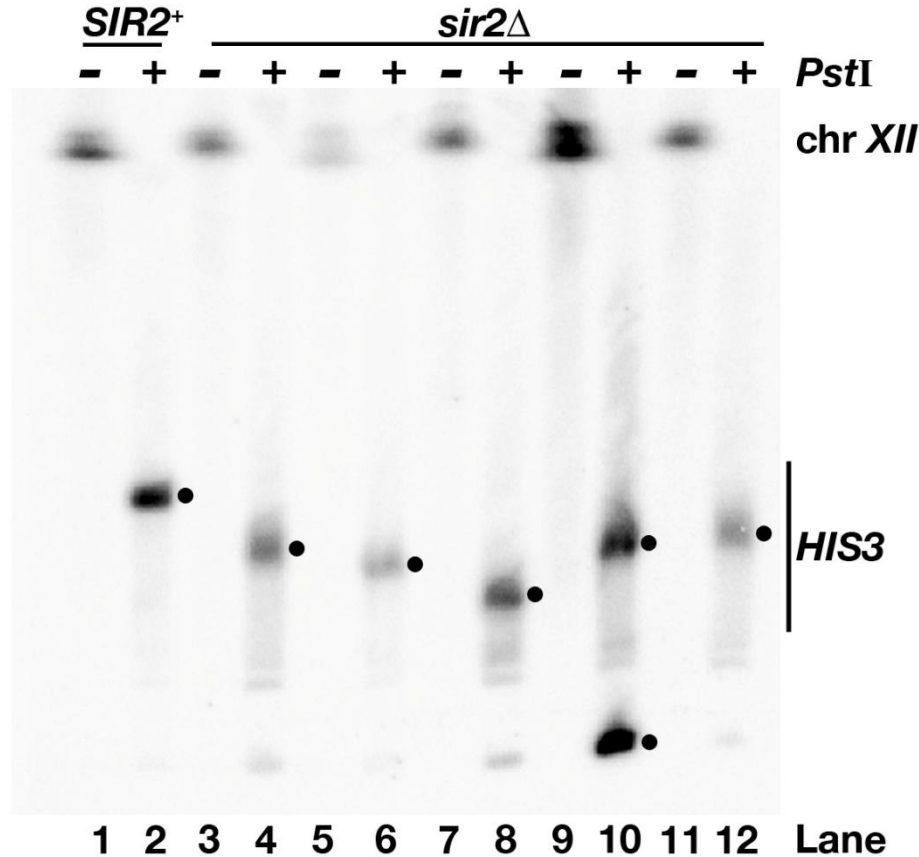


Figure 2-3. Loss of silent chromatin at the rDNA in *sir2*Δ cells causes movement of the *HIS3* reporter. The *HIS3* reporter gene integrated in NTS2 was mapped by *Pst*I digest of agarose-embedded chromosomes from five colonies of a *sir2*Δ strain (lanes 3-12) and compared to the *SIR2*⁺ parent (lanes 1-2). CHEF gel Southern blot with uncut and *Pst*I-digested chromosomal DNA was hybridized to a ³²P-labeled *HIS3* DNA probe. The *HIS3*-containing rDNA fragments in the *Pst*I cut samples are indicated by dots; the size difference between the smallest (lane 8) and largest (lane 12) *HIS3*-containing fragments is 17 rDNA repeat units. Uncut chr XII resolves as a doublet due to size (~1.845 kb). The *HIS3* probe anneals to two bands in lane 10, indicating that the *HIS3* gene in the rDNA array was duplicated in this *sir2*Δ colony.

2.3.2 Tet-Off regulation of *SIR2* allows for efficient depletion of the Sir2 protein

In order to study the loss and re-establishment of silent chromatin in the rDNA without rearrangement of the rDNA due to increased recombination, Sir2 needs to be depleted and reintroduced in a timely fashion. To accomplish this, a Tet-Off system was employed to regulate the expression of Sir2; depletion of Sir2 after addition of doxycycline to the growth media is expected to result in a loss of silent chromatin at the rDNA, and re-expression of Sir2 after the removal of doxycycline from the growth media, is expected to allow the re-establishment of silent chromatin at the rDNA.

In yeast strain MBY2508, which has a single *HIS3* reporter in the rDNA array, a tTA containing the strong transcriptional activator domain VP16 was integrated into the *ura3Δ0* locus to make MBY2776; because this strain has the endogenous *SIR2* promoter the addition of VP16-tTA should not alter *SIR2* expression. The *SIR2* endogenous promoter in MBY2776 was replaced with seven copies of the *tet* operator (*tetO₇-SIR2*) to make MBY2782; *SIR2* expression in this yeast strain is regulated by VP16-tTA. A time course was performed using MBY2782 to assess Sir2 protein levels in cells grown in the presence or absence of doxycycline to determine how long cells must grow in the presence of doxycycline in order to deplete Sir2 protein.

Two cultures of MBY2782 were grown simultaneously, one culture without doxycycline (No Dox) and one culture with 10 µg/ml doxycycline (+ Dox). Samples were taken every hour for five hours and used to prepare whole cell protein extracts. To confirm that neither the transcriptional activator (VP16-tTA) nor doxycycline altered the

level of Sir2 when its gene is under control of its endogenous promoter, two cultures of MBY2776 (VP16-tTA and endogenous *SIR2*), No Dox and + Dox, were analyzed in parallel. A Western blot was probed for Sir2 and a control protein, Pgk1. The level of Sir2 was normalized to Pgk1, and each Sir2/Pgk1 ratio was normalized to the Sir2/Pgk1 ratio of the 0 hour time point of MBY2782 No Dox culture. It was expected that Sir2 would deplete over time in the MBY2782 + Dox culture, while the MBY2782 No Dox culture would express Sir2 throughout the time course.

MBY2782 grown under No Dox conditions had Sir2 present throughout the time course as expected (Figure 2-4, lanes 1-6). There was an increase in Sir2 from hours 1-4 (Figure 2-4, lanes 2-5). MBY2782 grown under + Dox conditions showed a decrease in Sir2 beginning between 2-3 hours after addition of Dox; Sir2 was reduced to 10% of the starting level (0 hr) after five hours of growth (Figure 2-4, lanes 7-12). Sir2 levels in MBY2776, a strain with the VP16-tTA and the endogenous *SIR2* gene, were similar whether the strain was grown under No Dox or + Dox conditions for five hours, indicating that doxycycline does not alter Sir2 levels when *SIR2* is under control of its endogenous promoter (Figure 2-4, lanes 13-14). It is notable that there was a difference in the level of Sir2 protein in extracts from yeast strains MBY2782 and MBY2776. MBY2782 No Dox cells have a higher level of Sir2 protein at the 5 hour time point (Figure 2-4, lane 6) compared to MBY2776 (Figure 2-4, lanes 13 and 14). This indicates that cells containing *tetO₇-SIR2* + VP16-tTA overexpress *SIR2*. This experiment was repeated with a second, independent strain, and similar results were obtained (data not shown).

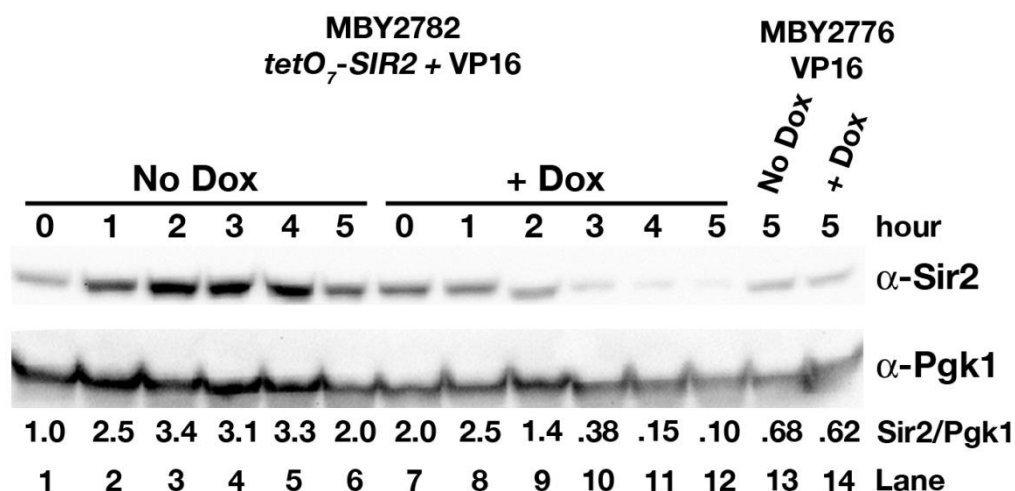


Figure 2-4. Addition of doxycycline results in depletion of Sir2. Duplicate cultures were grown with (+ Dox) or without doxycycline (No Dox) and whole cell extracts were prepared every hour for five hours to assess Sir2 levels by Western blot. Pgk1 serves as a loading control. Sir2/Pgk1 ratio was normalized to the No Dox 0 hour sample. Sir2 is present throughout the five hour time course in the No Dox culture of MBY2782. Sir2 is depleted to 10% of starting level after five hours growth with doxycycline. In a strain with endogenous *SIR2* promoter (MBY2776), doxycycline does not affect the level of Sir2 in the cells.

2.3.3 Comparison of Sir2 expression in strains with different Tet-Off systems

Sir2 is limiting for rDNA silent chromatin, meaning that overexpression of Sir2 leads to an increase in the strength of silent chromatin at the rDNA, and reduced expression of Sir2 leads to a decrease in the strength of silent chromatin at the rDNA [36]. Sir2 is overexpressed in cells with the *tetO₇-SIR2* + VP16 Tet-Off system (Figure 2-4). Because the level of Sir2 directly affects silent chromatin, a change in the level of Sir2 could alter the establishment of silent chromatin at the rDNA. Therefore, it is preferable to study the establishment of silent chromatin in cells that produce Sir2 protein at a level that is similar to that in wild-type cells.

The strength of expression of a gene under Tet-Off regulation can be altered by changing the number of *tet* operators in the *tetO* promoter and by changing the strength of the transcriptional activator domain in the tTA. To identify a condition in which the level of Sir2 in cells with Tet-Off regulation is similar to the level detected in wild-type cells two *tet* operators, *tetO₂* or *tetO₇*, were combined with one of two activators, VP16-tTA or Gcn4A-tTA. Decreasing the number of *tet* operators from seven to two decreases the number of binding sites for the tTA, which is expected to reduce activation of *SIR2* transcription. VP16 is a strong transcriptional activator and Gcn4A is a weaker transcriptional activator. By testing different combinations of *tet* operators and transcriptional activators, a specific combination that gives a level of Sir2 protein similar to that detected in wild-type cells should be obtained.

Our previous results with cells containing *tetO₇-SIR2* and VP16-tTA showed that Sir2 protein is present at a higher level than in cells with the endogenous *SIR2* gene (Figure 2-4). Therefore, the level of Sir2 protein was measured in cells where *tetO₇-SIR2* was combined with the weaker Gcn4A transcriptional activator, and *tetO₂-SIR2* was combined with either VP16-tTA or Gcn4A-tTA. Whole cell extracts were made from cultures grown to mid-log phase in rich media, without doxycycline. Western blot analysis was performed to measure the level of Sir2 protein in cells with different combinations of Tet-Off system components. The data show that cells containing *tetO₂-SIR2* with the Gcn4A-tTA had Sir2 levels most similar to wild-type cells (Figure 2-5). Cells with *tetO₇-SIR2* + Gcn4A-tTA had 5.2 to 6.6-fold more Sir2 than wild-type cells (Figure 2-5, lanes 3-5). Cells with *tetO₂-SIR2* + VP16-tTA had 2.3-fold more Sir2 than wild-type cells (Figure 2-5, lanes 6-7). Cells with *tetO₂-SIR2* + Gcn4A-tTA had a 1.9-fold increase in Sir2 protein compared to WT cells (Figure 2-5, lane 2). Three additional yeast strains containing *tetO₂-SIR2* + Gcn4A-tTA were tested by Western blot and had normalized Sir2/Pgk1 ratios of 0.60 to 2.2 (data not shown), supporting the initial result that this combination of Tet-Off system components produces Sir2 at levels similar to wild-type cells. Due to variability in the levels of Sir2 protein, preliminary Western blot analysis will be performed on extracts from newly constructed Tet-Off yeast strains to verify that the steady-state level of Sir2 protein is similar to that in wild-type cells.

2.3.4 The rDNA locus is stable in cells with *tetO₂-SIR2* + Gcn4A-tTA

Hyper-recombination in the rDNA can be assessed by comparing the location of the *HIS3* gene in individual colonies of a single strain. To verify stability of the *HIS3*

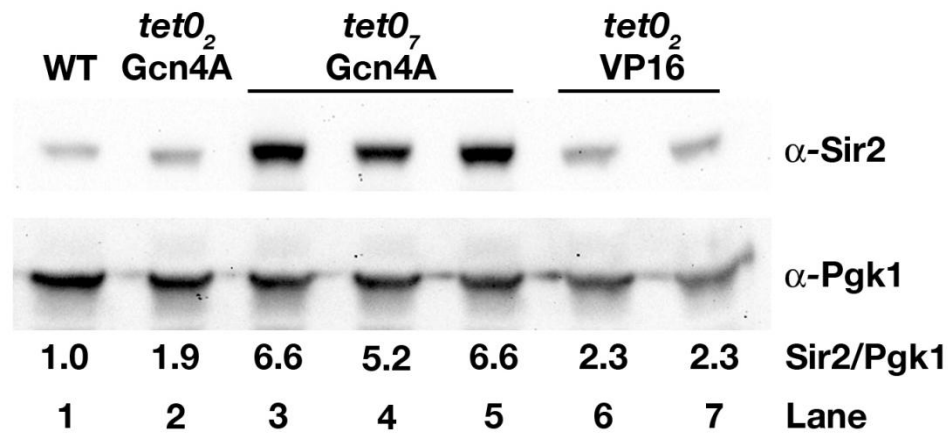
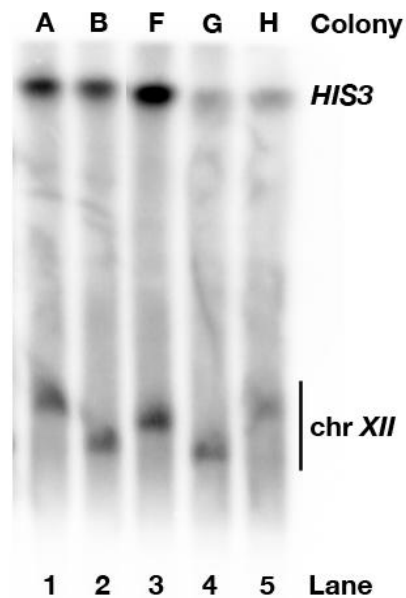


Figure 2-5. Tet-Off system with *tetO₂-SIR2* + Gcn4A-tTA has Sir2 levels most similar to wild-type (WT) cells. Strains with *SIR2* under control of different Tet-Off system components were compared by Western blot to determine which cells have levels of Sir2 protein most similar to wild-type cells. The level of Sir2 in *tetO₇-SIR2* + Gcn4A-tTA cells was 5.2- to 6.6-fold higher (lanes 3-5), and in *tetO₂-SIR2* + VP16-tTA cells (lanes 6-7) was 2.3-fold higher than WT (lane 1). Cells with *tetO₂-SIR2* + Gcn4A-tTA (lane 2) had Sir2 levels 1.9-fold higher than WT. The Sir2/Pgk1 ratio was normalized to the Sir2/Pgk1 ratio in the WT sample (lane 1).

gene within the rDNA array in strains with the Tet-Off system, colonies of MBY2851, a strain containing *tetO₂-SIR2* + Gcn4A-tTA, were analyzed as described above in Section 2.3.1. *Pst*I-digested chromosomes from individual colonies of MBY2851 were analyzed by CHEF gel Southern blot to compare the location of the *HIS3*-marked rDNA repeat unit within the rDNA array. *Pst*I-digested chromosomes were separated on a CHEF gel and the resulting Southern blot was analyzed using ³²P-labeled probes. A probe complementary to the 5' end of the *HIS3* gene was used to identify the *HIS3*-containing rDNA fragment and a probe complimentary to the NTS of the rDNA (Figure 2-6A) was used to identify both of the *Pst*I-generated rDNA fragments.

The *HIS3*-containing rDNA fragments were found to be of similar size in five of six colonies (Figure 2-6A); the *HIS3* gene had been duplicated in one colony (data not shown). The size difference between the smallest and largest *HIS3* fragment was approximately five rDNA repeat units. Size variation in the other rDNA fragment was approximately eleven rDNA repeat units. In other mapping experiments using sixteen different strains with a single *HIS3* gene integrated in the rDNA array, fragment size variation calculated from at least two independent mapping experiments was less than six rDNA repeat units (data not shown). While cells with the Tet-Off system have more variation in fragment size than what has been observed in cells with the endogenous *SIR2* gene, there is less variation in *HIS3*-fragment size in MBY2851 compared to the two *sir2Δ* strains described in Section 2.3.1, indicating the rDNA array in cells with the *SIR2* Tet-Off system is more stable than the rDNA array in *sir2Δ* cells.

A.



B.

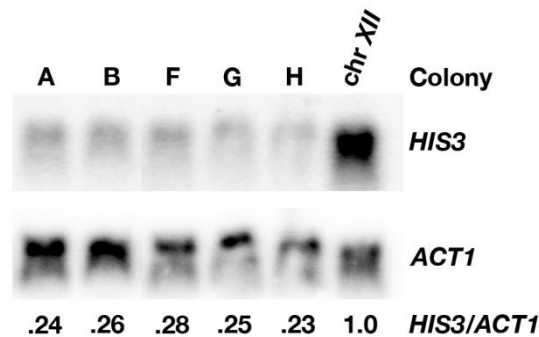


Figure 2-6. There is little movement of the *HIS3* gene within the rDNA array and similar levels of *HIS3* mRNA among colonies of the same Tet-Off strain. Five colonies of MBY2851 were analyzed to determine the location of the *HIS3* gene within the rDNA and the steady-state level of *HIS3* mRNA. (A) CHEF gel Southern blot of *Pst*I-digested agarose-embedded chromosomes hybridized to a probe complementary to the NTS of the rDNA. Fragments show little variation in size (variation of approximately five rDNA repeat units in the *HIS3*-marked fragment, approximately eleven rDNA repeat units in the *chr XII* fragment) among the five colonies, indicating minimal movement of the *HIS3*-marked rDNA repeat among colonies of the same strain. (B) Northern blot analysis of five colonies of MBY2851. *HIS3/ACT1* mRNA ratios were normalized to the *HIS3/ACT1* mRNA ratio from MBY2570, a strain having *HIS3* in a euchromatic region of *chr XII*. Normalized *HIS3/ACT1* mRNA ratios were similar from five colonies (0.23 to 0.28).

RNA from the five colonies of MBY2851 with a single *HIS3* gene in the rDNA was analyzed to assess Pol II transcriptional silencing. *ACT1* mRNA was used to normalize loading. *HIS3* mRNA/*ACT1* mRNA of each MBY2851 colony was normalized to a control strain (MBY2570) with a *HIS3* gene at a euchromatic location on chr *XII* outside of the rDNA. The normalized steady-state levels of *HIS3* mRNA from five colonies of MBY2851, 0.23 to 0.28, were similar (Figure 2-6B). Taken together, the results of the mapping and RNA analyses show that the rDNA in several colonies from the same *tetO₂-SIR2* + Gcn4A-tTA yeast strain is stable, with minimal movement of the *HIS3* gene in the rDNA, small differences in rDNA array size, and similar levels of silencing of the *HIS3* gene in the rDNA.

2.3.5 Sir2 is not detectable in a recovery time course

It was determined in time courses with MBY2782 that five hours of growth in the presence of 10 µg/ml doxycycline is sufficient to deplete Sir2 to 10% of the level in cells grown without doxycycline (Figure 2-4). To examine the depletion and recovery of Sir2 protein in cells with the *tetO₂-SIR2* + Gcn4A-tTA system, a time course was performed. Three strains were used: MBY2510 (no Tet-Off system, “WT”), MBY2933 (MBY2510 + Gcn4A-tTA), and MBY2952 (MBY2933 + *tetO₂-SIR2*). After six hours growth without (Figure 2-7A) or with (Figure 2-7B) doxycycline, cells were washed and grown in fresh media without doxycycline for 20 hours; after 20 hours, an aliquot of cells was diluted into fresh media and grown for 4 hours (Figure 2-7, “+4”).

Whole cell protein extracts from several time points were analyzed by Western blot to measure Sir2 protein levels. MBY2510 (WT) and MBY2933 (+ Gcn4A-tTA)

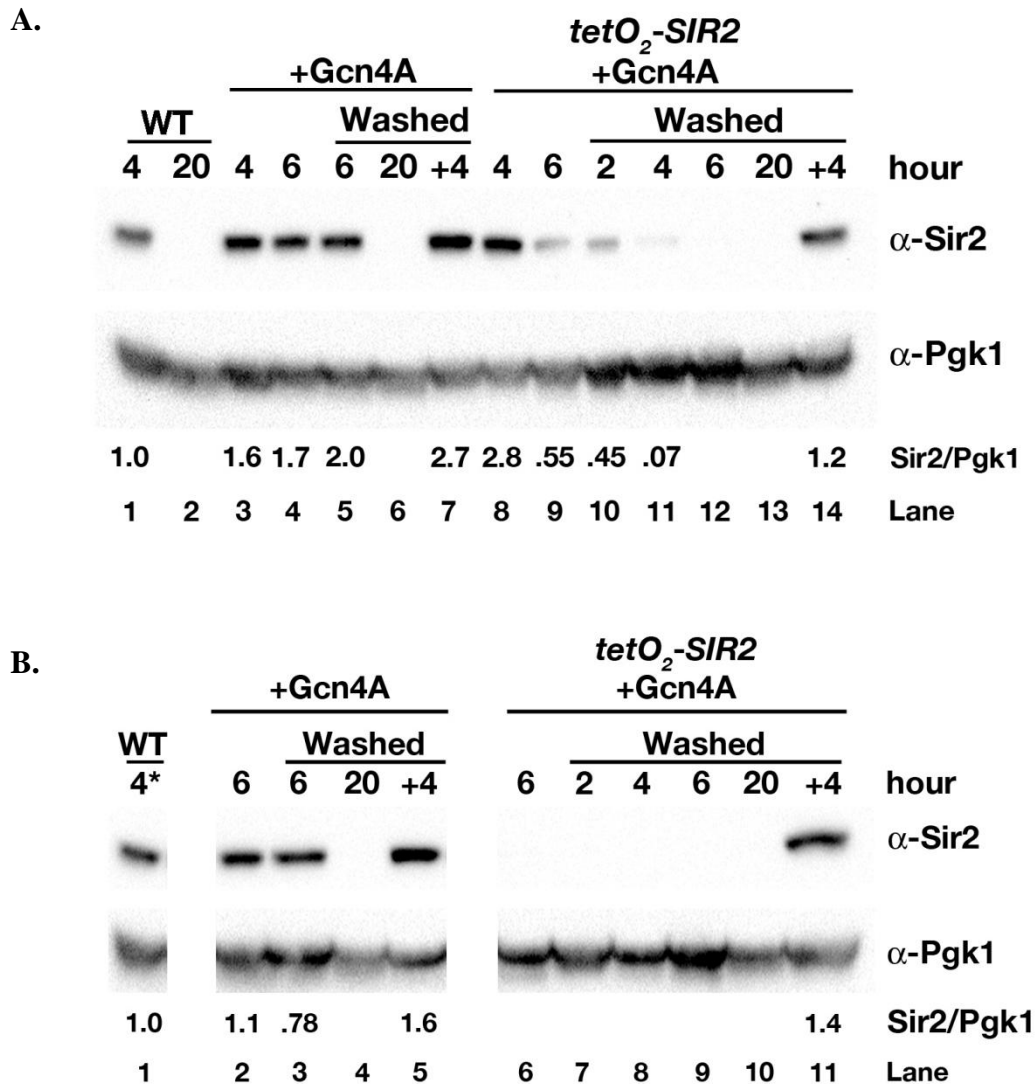


Figure 2-7. Sir2 is not detectable after 20 hours recovery growth. Cells were grown with or without doxycycline for six hours, washed, and grown in fresh media to allow for recovery of Sir2. Aliquots were taken during recovery growth after 2, 4, 6, and 20 hours; after 20 hours, cells were transferred to fresh media and grown four hours to mid-log phase (+4). Sir2/Pgk1 ratio was normalized to MBY2510 (“WT”). (A) Control recovery time course of cells grown in the absence of doxycycline during the six hour shut down. (B) Six hour shut down of Sir2 by the addition of 10 μ g/ml doxycycline prior to washing the cells and the recovery growth. No Sir2 was detected after six hours growth with doxycycline. Sir2 is not detectable after 20 hours recovery but is comparable to the level in WT cells after dilution and 4 hours of growth in fresh media. 4* indicates wild-type sample grown for 4 hours without doxycycline.

each contain the endogenous *SIR2* gene, and Sir2 protein was present at similar levels in both No Dox and + Dox cultures in all but one time point; Sir2 was not detectable in extracts from either strain after 20 hours of growth (Figure 2-7A, lanes 1-7; Figure 2-7B, lanes 1-5). One possibility is that *SIR2* expression is reduced in stationary-phase cultures. Results from a genome wide expression study showing that the expression of *SIR2* decreased over 12-fold (from 0 - 20.5hrs) during the diauxic shift [131] support this hypothesis. In a separate study, Sir2 was shown to be reduced substantially after 26 hours growth [125]. Further, the results in Figure 2-7A, lane 7, show that a near wild-type level of Sir2 is detected in whole cell extract from culture at mid log-phase that was started from the 20 hour recovery culture of MBY2933 (+ Gcn4A-tTA).

Results obtained from the No Dox time course experiment samples of MBY2952 (*tetO₂-SIR2* + Gcn4A-tTA) show that after six hours of growth in the absence of doxycycline, the level of Sir2 was reduced to about one third of the level in extracts from cells with the wild-type *SIR2* gene (Figure 2-7A, compare lane 9 to lane 4). The level of Sir2 protein in the MBY2952 No Dox culture continued to decrease even after the cells were washed and resuspended at low density ($\sim 4 \times 10^6$ cells/ml) in fresh media (Figure 2-7A, lanes 10-13). The reason for the decrease in the level of Sir2 in the Tet-Off cells grown without doxycycline is not known, but it is suspected that the Gcn4A-tTA component plays a role because reduction in Sir2 levels did not occur in cells with the endogenous promoter driving expression of *SIR2* at six hours (Figure 2-7A, lane 5).

Figure 2-7B shows the Western blot results for cultures grown in the presence of doxycycline. After six hours growth with doxycycline, Sir2 was not detectable in

extracts from MBY2952 (*tetO₂-SIR2* + Gcn4A-tTA), indicating that doxycycline-dependent shut down of *SIR2* transcription with the Tet-Off system was effective (Figure 2-7B, lane 6). However, after removal of doxycycline, the re-expression of Sir2 was not detected at several time points (2-20 hours) after the cells were washed and resuspended at low density ($\sim 4 \times 10^6$ /ml) in fresh media (Figure 2-7B, lanes 7-10). This result is consistent with the No Dox time course data shown in Figure 2-7A.

This experiment was repeated with another *tetO₂-SIR2* + Gcn4A-tTA strain (MBY2851) with the exception that recovery growth was allowed to continue for 22 hours as opposed to 20 hours; samples were taken from three time points during the time course and used to make chromosomal plugs so that the location of the *HIS3*-marked rDNA repeat unit within the rDNA array could be analyzed. The loss of Sir2 after treatment with doxycycline may allow for destabilization of the rDNA array, which can be assessed by movement of the *HIS3* gene in the rDNA. Movement of the *HIS3* gene after the depletion of Sir2 by doxycycline would indicate that yeast strains with the Tet-Off system do not maintain a stable rDNA array during depletion of Sir2. A Western blot was performed as described above (Figure 2-7) to analyze Sir2 levels during the time course; results similar to those in Figure 2-7 were obtained (data not shown). *Pst*I-digested chromosome plugs were made from samples taken throughout the time course: saturated starter culture, after six hours growth with or without doxycycline, and after six and 22 hours of growth following re-suspension of washed cells in fresh media without doxycycline. No change in rDNA fragment size was detected in any sample (Figure 2-8), indicating that the rDNA array remains stable in the absence of detectable Sir2 in the

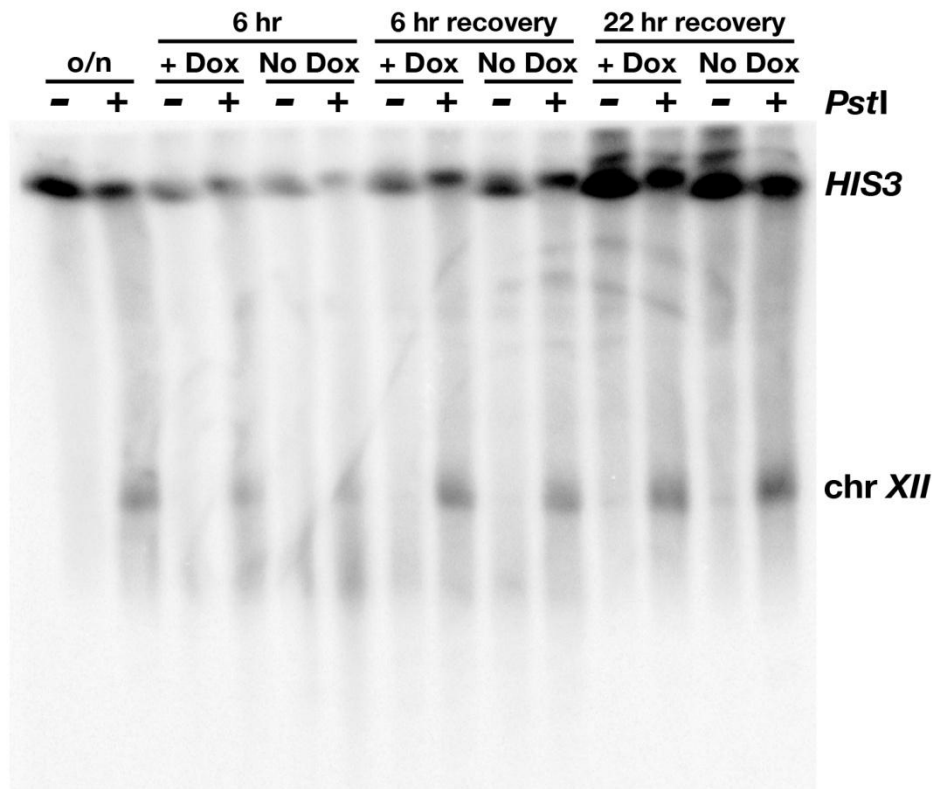


Figure 2-8. The *HIS3*-marked rDNA array is stable throughout a time course in a *SIR2* Tet-Off yeast strain. Cells were grown with or without doxycycline for six hours, washed, and grown in fresh media without doxycycline to allow for re-expression of *SIR2*. Samples were taken after 6 and 22 hours of recovery growth. CHEF gel Southern blot analysis of uncut and *PstI*-digested agarose embedded chromosomes was performed as described in Figure 2-6. Fragments of rDNA do not change in size during a time course when Sir2 is depleted after growth with doxycycline, indicating the rDNA array is stable when the Tet-Off system has repressed Sir2 expression. o/n, sample from a saturated (overnight) culture used to inoculate duplicate cultures for the time course.

time frame of these experiments. These promising data suggest it is possible to study the role of Sir2 in establishment of rDNA silent chromatin without hyper-recombination altering the rDNA array while Sir2 is absent from the cells.

2.4 DISCUSSION

While much is known about the establishment of silent chromatin at the *HM* loci and telomeres in *S. cerevisiae*, comparatively little is known about the establishment of silent chromatin at the rDNA [6, 69-71]. It has been shown by microscopy that Sir2 leaves the nucleolus, the home of the rDNA locus within the nucleus, during mitosis [37]. When Sir2 is absent or depleted from the cell, rDNA silent chromatin is compromised. Because Sir2 leaves the nucleolus during the cell cycle, it is expected that rDNA silent chromatin is compromised until Sir2 returns. Movement of the rDNA locus throughout the cell cycle has also been studied in live cells by microscopy [132], however changes in rDNA silent chromatin throughout the cell cycle have not yet been addressed. Our studies to examine rDNA silent chromatin during the cell cycle by assessing changes in Pol II transcriptional silencing at the rDNA in synchronized cell cultures were inconclusive (R. A. J., unpublished). The development of a system that allows the rapid depletion and re-expression of Sir2 is expected to facilitate studies into the mechanisms that regulate the establishment of silent chromatin at the rDNA.

2.4.1 A Tet-Off system to control expression of *SIR2*

The Tet-Off system originally developed for mammalian cells has been modified many times for use in yeast. Both the strength of the activator contained in the tTA and

the number of operators to which the tTA binds can affect the amount of transcription from the gene being controlled. To regulate expression of *SIR2*, we combined a strong (VP16-tTA) and a weak (Gcn4A-tTA) activator with two and seven copies of the *tet* operator; the combination of *tetO₂-SIR2* + Gcn4A-tTA was found to produce Sir2 levels most similar to that found in wild-type cells (Figures 2-4 and 2-5). This is important as the amount of Sir2 in the cell determines the strength of rDNA silent chromatin [36].

There is evidence that yeast cells with the *tetO₂-SIR2* + Gcn4A-tTA Tet-Off system do not produce a consistent level of Sir2 protein. After removing doxycycline from cultures, it was expected that Sir2 protein levels would increase; however, extracts from cultures grown for up to 20 hours after removal of doxycycline did not contain detectable Sir2 (Figure 2-7B). The lack of Sir2 was also seen in cultures grown without doxycycline, indicating the inability to detect Sir2 was not specific to the depletion of Sir2 by the addition of doxycycline. Without reliable expression of Sir2 and the ability to track the return of Sir2 in Tet-Off cultures, experiments to follow the re-establishment of rDNA silent chromatin as Sir2 accumulates will not be performed. Modifications to the *SIR2* Tet-Off system have been initiated by others in the Bryk lab.

2.4.2 Modifying the tTA

A common component of the VP16-tTA and Gcn4A-tTA is the promoter driving expression of the activator; the Cytomegalovirus (CMV) early promoter is present in both tTA constructs. One possibility is that the CMV promoter is not driving constitutive expression of the tTA in yeast strains, resulting in the observed decrease in Sir2 during growth without doxycycline. Our findings are not the first to hint that

expression of a tTA under CMV control in yeast is problematic. In experiments comparing gene expression from *tetO₂* and *tetO₇* promoters, the effect of adding five extra operator binding sites resulted in a mere two-fold increase in gene expression; it was proposed that the tTA driven by the CMV promoter is not expressed at a high level [112]. Additionally, in a tetracycline-regulatable dual system with both a tTA and a repressor protein intended to reduce basal transcription from the *tetO* promoter in the presence of doxycycline, changing the CMV promoter to the *S. pombe ADHI* promoter increased the effectiveness of the repressor. The substitution of the *ADHI* promoter enhanced the activity of Tup1, but not of a second repressor, Ssn6 [113]. These results suggest that replacing the CMV promoter may increase the expression of some, but not all, genes under control of the CMV promoter in *S. cerevisiae* cells. More recently it was reported that the CMV promoter is up-regulated in *S. cerevisiae* cells during osmotic stress and during a change in carbon source [133]. Although our cultures are grown in rich media under normal growth conditions, this observation suggests that the CMV promoter activity varies in a condition-dependent manner in *S. cerevisiae*. Construction of a modified version of the cassette driving expression of the tTA has been initiated; the CMV promoter driving expression of the Gcn4A-tTA activator will be replaced with the *S. cerevisiae PGK1* gene promoter, which is expressed at a consistent level in yeast cells.

Replacing the CMV promoter in the Gcn4A-tTA construct with a constitutive *S. cerevisiae* promoter should result in constitutive expression of *SIR2*. The promoter of *PGK1*, a well-characterized strong, constitutive promoter in *S. cerevisiae*, was used to

replace the CMV promoter in plasmid *pCMV-GCN4A-tTA* (MBB692). Experiments are underway with new Tet-Off yeast strains that contain the *PGK1-Gcn4A-tTA* and *tetO₂-SIR2* or *tetO₇-SIR2*. Extracts from these new strains will be analyzed by Western blot to determine if either strain expresses Sir2 protein at a level similar to that in wild-type cells. If Sir2 is expressed at wild-type levels, time courses in the absence of doxycycline will be performed to measure Sir2 levels to determine if Sir2 expression is constitutive; if Sir2 is expressed similar to Sir2 expression level in wild-type cells, these new Tet-Off yeast strains will be used to investigate the establishment of rDNA silent chromatin. If needed, other *S. cerevisiae* constitutive promoters, such as *PYK1*, *TEF1*, or *ADH1*, which have all been used as constitutive promoters in other expression vector systems [134], will be tested to achieve stable expression of Sir2 at wild-type levels in yeast strains with the Tet-Off system.

2.4.3 Future use of a Tet-Off system to study rDNA silent chromatin

The ultimate goal of using a Tet-Off system to control *SIR2* expression is to track the establishment of rDNA silent chromatin as Sir2 is re-expressed after being depleted. ChIP experiments during recovery of Sir2 will analyze the composition of rDNA silent chromatin across the entire rDNA array and within a single *HIS3*-marked rDNA repeat as silent chromatin is re-established during the accumulation of Sir2 in the cells. The levels of acetylated and methylated histones will be analyzed by ChIP, and are expected to decrease as Sir2 accumulates. Sir2 is a histone deacetylase, so it is expected that as Sir2 association increases at the rDNA, the level of acetylated histone H3 will decrease. Based on previous reports assessing the levels of methylated histone H3 in *SIR2*⁺

compared to *sir2Δ* cells [18], it is expected that as acetylated histone H3 decreases, levels of methylated histone H3 will also decrease.

During the Sir2-dependent change in rDNA chromatin structure, ChIP experiments to assess the association of other known silencing proteins will shed light on the establishment of rDNA silent chromatin. Net1 is an rDNA-associated protein that recruits Sir2 to the rDNA and plays a role in nucleolar integrity [37, 39]. A tagged allele of *NET1* has been used previously in ChIPs to assess binding of Net1 to the rDNA array [22]; tracking Net1 association with the rDNA as Sir2 levels change could add to the current knowledge about Sir2-Net1 interactions as well as Net1-rDNA interactions and how Net1 acts to preserve nucleolar integrity. The development of the Tet-Off system is an important tool needed to learn more about the establishment of silent chromatin at the rDNA; a better understanding of how rDNA silent chromatin is formed can lead to advances in the knowledge of the mechanisms that regulate highly repeated DNA sequences in eukaryotic cells.

III. DYNAMIC REGULATION OF SILENT CHROMATIN WITHIN THE RIBOSOMAL DNA LOCUS OF *Saccharomyces cerevisiae*

3.1 INTRODUCTION

The ribosomal DNA (rDNA) locus of *Saccharomyces cerevisiae* is, like in many eukaryotes, a highly repeated locus. The rDNA is ~1.8 megabases on chromosome *XII*, comprised of ~200 copies of tandemly arranged rDNA repeats. Each rDNA repeat unit contains a Pol I transcribed 35S rRNA gene and a Pol III transcribed 5S rRNA gene that divides a non-transcribed spacer region (NTS) into two parts, NTS1 and NTS2. Pol II transcribed genes located within the rDNA are silenced [26-28], meaning Pol II transcription is repressed due to silent chromatin that forms across the rDNA locus. Silent chromatin also stabilizes this large, highly repeated locus by repressing homologous recombination between rDNA repeats [29, 66].

Silent chromatin exists in three regions in the *S. cerevisiae* genome: the *HM* loci, telomeres, and rDNA locus [6, 69-71]. Variation in the strength of silent chromatin has been documented at the telomeres and is termed telomere position effect [74]. However, the silent chromatin responsible for repressing transcription and recombination in the rDNA repeats has been assumed to be uniform across the ~1.8 Mb rDNA array. In previous studies it was observed that Ty1 retrotransposons integrated into the rDNA were expressed at different levels [26, 28], hinting that silent chromatin in the rDNA is not uniform. A caveat that precluded clear interpretation of this possibility is that the position of the Ty1 elements within each rDNA repeat was different, so the flanking

DNA sequences were also different and may have contributed to variation in the level of Ty1 element expression.

To determine if silent chromatin in different rDNA repeat units is uniform, a collection of yeast strains was constructed so that each strain contains a single copy of the *HIS3* gene integrated at the same DNA sequence within an rDNA repeat unit but located in a different repeat unit within the rDNA locus. Measurement of mRNA levels from the Pol II transcribed gene *HIS3*, which is silenced when placed in the rDNA array, and the rates of mitotic recombination revealed variation in the level of steady-state *HIS3* mRNA and the rate of mitotic homologous recombination among the yeast strains in the collection. These data indicate that silent chromatin is not equivalent in individual rDNA repeats across the rDNA locus. Variability in silent chromatin at the rDNA is called rDNA Position Effect (rDNA-PE).

The results presented here support the existence of different chromatin states within the rDNA array. Previous work has shown that the Pol I-transcribed 35S rRNA genes exist in two disparate chromatin states; psoralen accessible, or transcriptionally active, and psoralen resistant, transcriptionally silent [7]. Additionally, the level of Pol I transcription has been shown to correlate inversely with transcriptional silencing of Pol II genes in the rDNA [55]. When Pol I transcription of the 35S rRNA genes is impaired, Pol II transcription in the rDNA is increased [53, 55]. Considering these observations, finding variation in silent chromatin in different rDNA repeat units is not entirely unexpected; however, until now the effect and mechanism of this variability in rDNA silent chromatin has not been investigated.

Modified histones have a documented role in rDNA silent chromatin. The NAD⁺-dependent histone deacetylase Sir2 is a member of the protein complex RENT (regulator of nucleolar silencing and telophase exit) [31, 37] and its activity is required for rDNA silent chromatin to repress both Pol II transcription and homologous recombination by preventing the accumulation of acetylated histone H3 [26, 28-30]. Another component of RENT is Net1, which functions solely at the rDNA to recruit Sir2 to specific regions of rDNA repeat units [37]. Chromatin immunoprecipitation experiments have shown that Sir2 and Net1 associate with two regions of the rDNA repeat unit, in NTS1 at the replication fork barrier and in NTS2 at the promoter region of the 35S rRNA gene [22]. When the *SIR2* or *NET1* genes are deleted, rDNA silent chromatin does not form, resulting in higher levels of Pol II transcription and homologous recombination in the rDNA that contribute to the instability of the locus to the detriment of the cell [25, 26, 28, 29, 37, 40]. Cdc14 is the third RENT member; it is sequestered in the nucleolus and regulates the cell's exit from mitosis; however, the role of Cdc14 in rDNA silent chromatin remains unknown [38].

In addition to the RENT complex and histone acetylation, the histone methyltransferase Set1 has been shown to regulate rDNA silent chromatin [35, 135]. Set1 is one protein of the eight-member complex COMPASS, and is responsible for mono-, di-, and tri-methylation of lysine 4 on the tail of histone H3 (H3K4) in *S. cerevisiae* [41, 136]. Set1 is required for Pol II transcriptional silencing, but, unlike Sir2, does not play a critical role in the repression of homologous recombination at the

rDNA. Sir2 and Set1 act independently in rDNA silent chromatin, however the loss of Sir2 results in increased H3K4 methylation at the rDNA [18, 35].

How factors that regulate rDNA silent chromatin affect rDNA-PE has been investigated using *set1Δ* strains and chromatin immunoprecipitation experiments. Deletion of Set1 resulted in a loss of transcriptional silencing at the rDNA in most strains, yet variation in the levels of Pol II transcript from different rDNA repeats was still detected. ChIP experiments to assess the level of Sir2 protein with rDNA repeats revealed that less Sir2 is associated with more highly expressed rDNA repeats. These data indicate that silent chromatin in rDNA repeats is not uniform. Moreover, the results suggest that rDNA repeat units are differentially regulated, with Sir2 playing a primary role in the strength of silent chromatin within a single rDNA repeat. Studying this new property of rDNA silent chromatin will lead to a better understanding of the epigenetic regulation of repeated DNA sequences.

3.2 MATERIALS AND METHODS

3.2.1 Media

Growth media used in these experiments has been described previously [123]. YPADT is YPD media supplemented with 40 mg/L adenine hemisulfate and 80 mg/L L-tryptophan. YPADTU is YPADT media supplemented with 20 mg/L uracil. When indicated, doxycycline was added to media at a concentration of 10 µg/ml.

3.2.2 Construction of yeast strain collections

All *S. cerevisiae* strains constructed and used in this study are listed in Table 3-1.

To generate collections of yeast strains with a reporter gene in the rDNA array, a fragment containing the *HIS3* gene was amplified from pRS403 and integrated into the rDNA locus of yeast strain MBY1202 by integrative lithium acetate transformation [124] with the following modifications. Saturated cultures grown in YPADT or YPADTU were diluted into fresh YPADT or YPADTU media to $\sim 4 \times 10^6$ cells/ml and grown to $1-2 \times 10^7$ cells/ml. Cells were harvested by centrifugation and washed with 10 ml sterile water, then resuspended in 1 ml 1X TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0)/0.1 M Lithium acetate (LiAc) and centrifuged 2 minutes at 5k rpm. Cells were resuspended in 100 μ l 1X TE/0.1 M LiAc per 20 ml original culture. Aliquots (100 μ l) were incubated with 50 μ g carrier DNA and 1-10 μ g transforming DNA at 30°C for 30 minutes, followed by incubation at 30°C for 30 minutes with 40% Polyethylene glycol 3350/1X TE/0.1M LiAc. Cells were heat shocked at 42°C for 15 minutes, washed with sterile water, plated on selective media and grown at 30°C for 2-3 days, until single colonies appeared. In strains using a *KANMX* marker for gene replacement and transformant selection, cells were incubated in 2 ml YPADT or YPADTU at 30°C for an additional two hours prior to plating on selective media containing 200 mg/L G418. The *TRP1* reporter gene was amplified from pRS404. The resulting fragment was used in an integrative lithium acetate transformation as described above to replace the *HIS3* gene in the rDNA by homologous recombination. The position of the reporter gene within the

Table 3-1. Strains used in this study.

Name	Genotype	Reporter Location		# rDNA repeats
		kb to CEN ^a	kb to TEL ^a	
MBY1202	<i>MATα his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0</i>			
MBY2506	MBY1202 NTS2:: <i>HIS3</i>	1183	327	166
MBY2507	MBY1202 NTS2:: <i>HIS3</i>	25	1173	132
MBY2508	MBY1202 NTS2:: <i>HIS3</i>	321	1036	149
MBY2509	MBY1202 NTS2:: <i>HIS3</i>	1116	396	166
MBY2510	MBY1202 NTS2:: <i>HIS3</i>	725	809	168
MBY2570	MBY1202 <i>chr XII</i> :: <i>HIS3</i>			
MBY2603	MBY1202 NTS2:: <i>HIS3</i>	1151	454	176
MBY2604	MBY1202 NTS2:: <i>HIS3</i>	1011	129	125
MBY2607	MBY1202 NTS2:: <i>HIS3</i>	<48	1106	<127
MBY2608	MBY1202 NTS2:: <i>HIS3</i>	959	1049	221
MBY2609	MBY1202 NTS2:: <i>HIS3</i>	183	1149	146
MBY2611	MBY1202 NTS2:: <i>HIS3</i>	1003	359	150
MBY2613	MBY1202 NTS2:: <i>HIS3</i>	507	879	152
MBY2614	MBY1202 NTS2:: <i>HIS3</i>	1147	255	154
MBY2616	MBY1202 NTS2:: <i>HIS3</i>	473	898	151
MBY2617	MBY1202 NTS2:: <i>HIS3</i>	1184	409	175
MBY2619	MBY1202 NTS2:: <i>HIS3</i>	1104	603	122
MBY2620	MBY1202 NTS1:: <i>HIS3</i>	335	936	140
MBY2622	MBY1202 NTS1:: <i>HIS3</i>	895	546	158
MBY2624	MBY1202 NTS1:: <i>HIS3</i>	87	1221	144
MBY2625	MBY1202 NTS1:: <i>HIS3</i>	160	1122	141
MBY2626	MBY1202 NTS1:: <i>HIS3</i>	749	1018	194
MBY2627	MBY1202 NTS1:: <i>HIS3</i>	117	1129	136
MBY2628	MBY1202 NTS1:: <i>HIS3</i>	476	893	150
MBY2630	MBY1202 NTS1:: <i>HIS3</i>	949	259	135
MBY2636	MBY1202 NTS1:: <i>HIS3</i>	1096	279	151
MBY2724	MBY2665 NTS2:: <i>rHIS3</i>	1182	106	142
MBY2731	MBY2665 NTS2:: <i>rHIS3</i>	200	1105	143
MBY2733	MBY2665 NTS2:: <i>rHIS3</i>	1179	<48	<135
MBY2760	MBY2665 NTS2:: <i>rHIS3</i>	900	605	165
MBY2761	MBY2665 NTS2:: <i>rHIS3</i>	429	1136	172
MBY2766	MBY2665 NTS2:: <i>rHIS3</i>	1121	443	172
MBY2768	MBY2665 NTS2:: <i>rHIS3</i>	1149	<48	<132

Table 3-1 Continued.

Name	Genotype	Reporter Location		# rDNA repeats
		kb to CEN ^a	kb to TEL ^a	
MBY2851	MBY2603 chr <i>I</i> ::pUI-tTA-GCN4A- <i>ADHI</i> _{term} - <i>URA3 KANMX6-tetO</i> ₂ - <i>CYC1</i> _{TATA} :: <i>SIR2</i>			
MBY2853	MBY2570 chr <i>XII</i> :: <i>his3</i> :: <i>TRP1</i>			
MBY2855	MBY2508 NTS2:: <i>his3</i> :: <i>TRP1</i>	<48 ^b	1159 ^b	<133
MBY2856	MBY2608 NTS2:: <i>his3</i> :: <i>TRP1</i>	1188 ^b	689 ^b	206
MBY2857	MBY2617 NTS2:: <i>his3</i> :: <i>TRP1</i>	1203 ^b	59 ^b	139
MBY2807	MBY2570 <i>set1</i> Δ:: <i>KANMX4</i>			
MBY2811	MBY2508 <i>set1</i> Δ:: <i>KANMX4</i>	496	1070	172
MBY2867	MBY2603 <i>set1</i> Δ:: <i>KANMX4</i>	1111	470	174
MBY2868	MBY2603 <i>set1</i> Δ:: <i>KANMX4</i>	1126	486	177
MBY2869	MBY2617 <i>set1</i> Δ:: <i>KANMX4</i>	1111	394	165
MBY2870	MBY2617 <i>set1</i> Δ:: <i>KANMX4</i>	1019	379	154
MBY2948	MBY2507 chr <i>I</i> ::pUI-tTA-GCN4A- <i>ADHI</i> _{term} - <i>URA3 KANMX6-tetO</i> ₂ - <i>CYC1</i> _{TATA} :: <i>SIR2</i>			
MBY2951	MBY2509 chr <i>I</i> ::pUI-tTA-GCN4A- <i>ADHI</i> _{term} - <i>URA3 KANMX6-tetO</i> ₂ - <i>CYC1</i> _{TATA} :: <i>SIR2</i>			
MBY2954	MBY2608 chr <i>I</i> ::pUI-tTA-GCN4A- <i>ADHI</i> _{term} - <i>URA3 KANMX6-tetO</i> ₂ - <i>CYC1</i> _{TATA} :: <i>SIR2</i>			
MBY2961	MBY2608 <i>set1</i> Δ:: <i>KANMX4</i>	849	1087	212
MBY2962	MBY2608 <i>set1</i> Δ:: <i>KANMX4</i>	929	1135	226

^akilobases between reporter location and the centromere (CEN) or telomere (TEL) promimal side of the rDNA array.

^b*TRP1* probe hybridizes to both fragments of *Pst*I-digested rDNA, therefore the relative position of the marker to the centromere or telomere-proximal side of the rDNA array cannot be determined.

rDNA array in each strain was determined by separating digested chromosomes and a lambda concatemer ladder by CHEF gel electrophoresis.

3.2.3 CHEF gel analysis and mapping of a single reporter in the rDNA array

Experiments were performed as previously described [26, 127] with the following modifications. Chromosomal DNA embedded in low melt agarose plugs was prepared from saturated cultures grown in YPADT or YPADTU. Plugs were incubated with 1 mM phenylmethylsulfonyl fluoride (PMSF) and washed extensively prior to digestion with 40-80 units *Pst*I (New England Biolabs) at 37°C overnight. For most experiments undigested plugs incubated without restriction enzyme were also analyzed. Chromosomal DNA and a lambda PFG ladder (New England Biolabs) were separated by electrophoresis using a BioRad CHEF mapper on a 1% agarose gel in 0.5X TBE buffer at 14°C, using 6 V/cm 60 second pulses for 15 hours followed by 6 V/cm 90 second pulses for 9 hours at an angle of 120°. The gel was stained with ethidium bromide and imaged. After 2 minutes of exposure to UV light in a Stratalinker 2400 (Stratagene) to nick the DNA fragments, Southern blotting was performed as described below. Membranes were hybridized first to ³²P-labeled DNA probes made by random priming using the *HIS3* gene as a template. Blots were imaged using a Pharos FX Plus Molecular Imager (BioRad). After imaging, the membrane was stripped for two hours at 70°C with 50% formamide in 1X SSC (0.15 M NaCl, 0.015 M HOC(COONa)(CH₂COONa)•2H₂O, pH 7.0-7.5). The membrane was then incubated with a ³²P-labeled DNA probe made by random priming using the rDNA NTS as a template. After imaging, the sizes of labeled rDNA fragments were estimated from the

lambda PFG ladder, which were then used to determine the location of *HIS3* gene within the rDNA array and the length of the rDNA array. For each strain, the mapping of the *HIS3* reporter in the NTS2 region of the rDNA was performed at least twice on separate CHEF gels, using different isolates of each strain. The location of the *HIS3* gene in the replicate experiments was compared to determine the reproducibility of mapping; the average difference in fragment size between replicate experiments was less than six repeat units. This indicates that individual mapping experiments reliably provide a consistent location of the *HIS3*-marked rDNA repeat within the rDNA array and that movement of the *HIS3*-marked rDNA repeat, when detected, is not likely contributable to mapping error.

The position of the *TRP1* gene was mapped by CHEF gel Southern blot, as described above, except that the restriction digest was performed with 50 units *BstXI* (New England Biolabs) and a ³²P-labeled probe complementary to *TRP1* was used. The *TRP1* probe anneals to both pieces of the rDNA after digestion with *BstXI*. Therefore, the CHEF gel analysis can be used to determine if a single *TRP1* gene is present but cannot determine the exact position of the reporter relative to the centromere- or telomere-proximal side of the rDNA array.

3.2.4 Southern blot analysis

Southern blot analysis of *SfiI*-digested genomic DNA was used to verify that multimers of the *HIS3* gene were not present in the rDNA (data not shown). Genomic DNA was digested with 30 units *SfiI* (New England Biolabs) for 12-16 hours at 50°C. DNA fragments were separated on a 1% agarose gel in 1X TAE buffer by

electrophoresis. Gels were incubated with shaking at room temperature for 30 minutes each in denaturation solution (0.2 N NaOH, 0.6 M NaCl), followed by neutralization solution (1.5 M NaCl, 1 M Tris, pH 7.4) and 10X SSC (1.5 M NaCl, 0.15 M HOC(COONa)(CH₂COONa)•2H₂O, pH 7.0-7.5). DNA was transferred to Genescreen, Hybond, or Hybond-XL membrane (Amersham) by blotting with 10X SSC for 12-16 hours. ³²P-labeled probes used in blotting experiments were synthesized by random priming.

3.2.5 Northern blot analysis

Cultures of yeast strains grown overnight to saturation in YPADT or YPADTU were diluted into fresh YPADT or YPADTU at ~4x10⁶ cells/ml and grown at 30°C to a density of 1-2x10⁷ cells/ml. Total RNA was extracted from yeast cultures as previously described [129] with the following modifications. Cells were resuspended in 450 µl AE buffer (50 mM C₂H₃NaO₂ pH 5.3, 10 mM EDTA) transferred to a microfuge tube, and 50 µl 10% SDS was added. An extraction with an equal volume of chloroform:isoamyl alcohol (24:1) was done before the addition of 50 µl 3M sodium acetate pH 5.3 and 2.5 volumes 100% ethanol. After precipitation RNA was resuspended in 20-30 µl sterile water and stored at -70°C. RNA (15-30 µg) was analyzed by Northern blot as previously described [130]. ³²P-labelled DNA probes synthesized by random priming were hybridized to membranes to measure the steady state level of *HIS3* or *TRP1* transcript. A ³²P-labelled DNA probe specific for *ACT1* was used as a loading control.

3.2.6 Recombination assay to measure mitotic stability of the *HIS3* reporter

Experiments were performed as previously described [26].

3.2.7 Whole cell extract and Western blot analysis

Whole cell protein extracts were prepared as described previously [128]; in some experiments 20-25 ml cells were harvested and 0.125 ml RIPA buffer was used. Protein from whole cell extracts (30 µg, 80 µg, or 90 µg) was separated on 10% SDS-PAGE, transferred to PVDF membrane and probed with α -Sir2 yN-19 (sc-6666, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:1000). Antibody binding was detected with IgG-HRP-conjugated secondary antibody donkey α -goat (sc-2020, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:15,000) and Clarity Western ECL Substrate (Bio-Rad, Hercules, CA). As a loading control in experiments using the Tet-off system, the membrane was probed with α -phosphoglycerate kinase (Pgk1) primary antibody (459250, Invitrogen, Camarillo, CA; 1:2000) and detected with IgG-HRP conjugated α -mouse secondary antibody (W402B, Promega, Madison, WI; 1:15,000) and Clarity Western ECL Substrate. Blots were imaged with Bio-Rad Molecular Imager ChemiDoc XRS and quantified with Quantity One software.

3.2.8 Chromatin immunoprecipitation

Cultures of yeast cells were grown at 30°C to $1.5-3 \times 10^7$ cells/ml in 150 ml YPADT or YPADTU. Lysates were prepared as previously described [137] with the following modifications. Breakage of cells was performed in 500 µl lysis buffer (50 mM HEPES/KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium deoxycholate, 1 mM PMSF, 1 mM benzamidine, 1 µg/ml each leupeptin,

pepstatin, and bestatin) using a Mini-BeadBeater 16 (Biospec) at 4°C, 1 minute beating followed by 2 minutes of rest, repeated three times. Chromatin in 1 ml lysis buffer was sonicated in a 4°C waterbath (Bioruptor Water Cooler, Diagenode) using a Bioruptor 300 Sonication System (Diagenode) for 80-120 cycles of 30 seconds on, 45 seconds off, power setting high, to shear chromatin to a length <1,000 base pairs. Sonicated chromatin was clarified by centrifugation at 14K rpm, 4°C, 30 minutes. For each ChIP, sonicated chromatin was incubated with antibody in a total volume of 500 µl overnight, rocking at 4°C. Sir2 ChIPs used 250 µl chromatin and 15 µl anti-Sir2 yN-19 (sc-6666, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibody. K9K14AcH3 ChIPs used 200 µl chromatin and 10 µl anti-K9K14AcH3 (#06-599, Upstate Cell Signaling Solution, Lake Placid, NY) antibody.

3.2.9 Analysis of ChIPs

Quantitative Polymerase Chain Reactions (qPCR) were performed to analyze the association of the Sir2 or acetylated histone H3 in rDNA repeats. To analyze the *HIS3*-marked rDNA repeats, triplicate reactions using 5 µl input DNA (1:10) and 5 µl immunoprecipitated DNA were amplified in 20 µl reactions containing 0.5 µM each oligonucleotide and 1X homemade master mix (1X Apex PCR buffer, 2.5 mM MgCl₂, 0.2 mM dNTPs, 1X Evagreen (Biotium), and 1 unit Apex Taq). To measure the average level of Sir2 associated with unmarked rDNA repeats, triplicate reactions using 1 µl input DNA (1:100) and 1 µl immunoprecipitated DNA (1:10) were amplified in 20 µl reactions containing 1.25 µM each oligonucleotide and 1X homemade master mix. Reactions were performed in a BioRad CFX96 Real-Time System C1000 Thermal

Cycler. The PCR parameters were 1 cycle of 95°C, 3 minutes; 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds; 1 cycle of 95°C for 30 seconds followed by melt curve analysis from 65°C to 95°C in 0.5°C increments for 5 seconds each. The threshold cycle reading was taken after each 72°C elongation step. Percentage of DNA immunoprecipitated (%IP) was calculated by dividing the signal from IP DNA by that of input DNA. %IP from no antibody samples was subtracted as background. %IP at the *HIS3*-marked rDNA repeat was normalized to the %IP of unmarked rDNA repeats.

3.2.10 Oligonucleotides for ChIP

Oligonucleotide sequences are listed in Table 3-2. To analyze the *HIS3*-marked repeat within the rDNA, a primer that anneals in the promoter of *HIS3* (OM1124) was used with an rDNA-specific primer (OM443 or OM451). To analyze association with unmarked rDNA repeats, two rDNA-specific primers were used that amplify each rDNA repeat unit at the location in NTS1 (OM442 and OM443) or NTS2 (OM450 and OM451) of *HIS3* integration. Oligonucleotides used to analyze the rDNA at have been described previously [18, 22]. Primers to amplify a region of the *RPS16A* gene and an intergenic region on chromosome *VIII* for K9K14AcH3 ChIPs were described previously [18, 138].

3.2.11 Growth with doxycycline

Saturated cultures were used to inoculate duplicate cultures at $\sim 4 \times 10^6$ cells/ml in YPADTU. Doxycycline was added from a 5 mg/ml stock solution in sterile water to one of the duplicate cultures at a concentration of 10 μ g/ml. Cultures were grown for six

Table 3-2. Oligonucleotides used in this study.

Name	Sequence
OM 442	TGATGATGGCAAGTTCCAGA
OM 443	CTTATTCCTTCCCGCTTTCC
OM 450	TGCAAAAGACAAATGGATGG
OM 451	GCACCTTTTCCTCTGTCCAC
OM 1124	GTACTAGAGGAGGCCAAG

hours shaking at ~250-300 rpm on a platform shaker at 30°C. Cell cultures (20 ml) were harvested for RNA extraction (described in Section 3.2.5) after six hours; in most experiments, 20 ml of culture were harvested for whole cell protein extract (described in Section 3.2.7) and 1-10 ml of culture was used to prepare agarose-embedded chromosome plugs (described in Section 3.2.3). To remove doxycycline from cultures, cells were washed three times with 1 ml sterile water, then single colony purified to YPADTU agar plates and grown at 30°C for 2-3 days, until single colonies appeared. Individual colonies were patched to YPADTU agar plates and analyzed by Northern blot as described above; in most experiments colonies were also analyzed by Western blot and CHEF gel Southern blot as described above.

3.3 RESULTS

3.3.1 Integration and mapping of *HIS3* in the rDNA NTS

Introduction of a Pol II transcribed reporter gene into the ribosomal DNA locus, such as the *S. cerevisiae HIS3* gene, is used routinely to assess silent chromatin in the rDNA. Many studies have made conclusions about silent chromatin in the rDNA based on data from a single yeast strain with a single reporter gene in the rDNA. Because the rDNA locus is ~1.8 megabases in length, it is possible that silent chromatin may not be identical across the locus. Here, experiments were performed to address the uniformity of silent chromatin across the ~1.8 megabase rDNA array using a collection of yeast strains, where each strain has a *HIS3* gene integrated at the same position within an rDNA repeat unit but in different positions across the rDNA locus (Figure 3-1). To

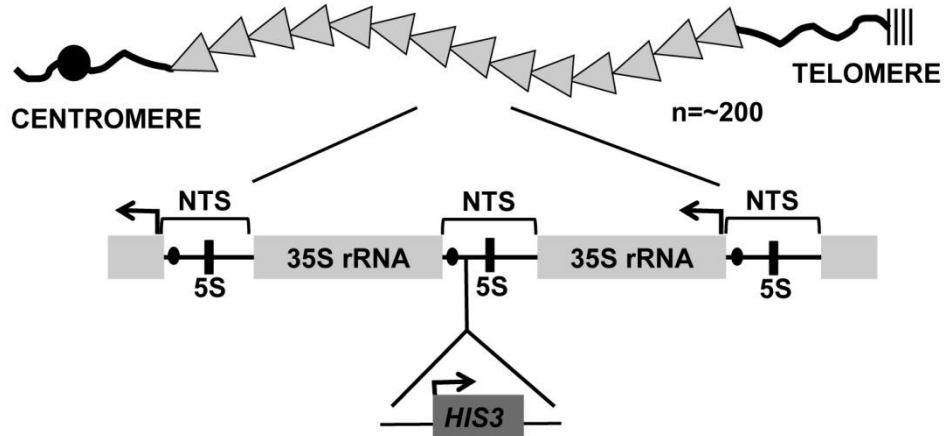


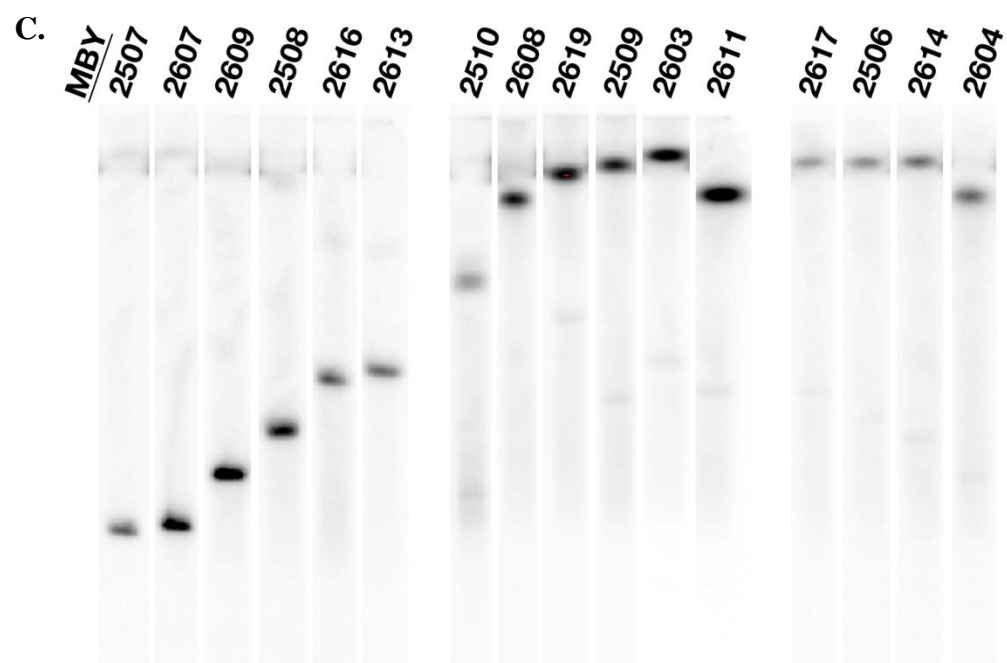
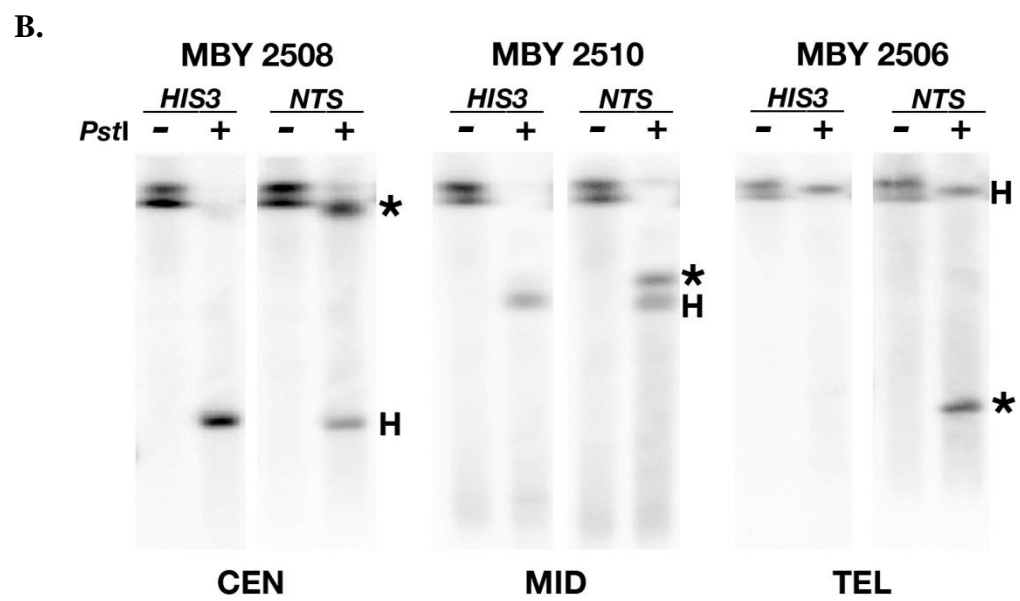
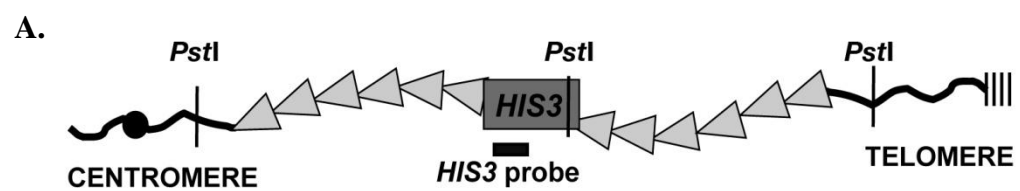
Figure 3-1. Structure of the rDNA locus in *S. cerevisiae* and integration of the *HIS3* gene. Triangles represent the ~200 rDNA repeats on chromosome *XII*. Each repeat contains the 35S rRNA gene and a non-transcribed spacer (NTS) region divided into NTS1 and NTS2 by the 5S rRNA gene. The *HIS3* gene is shown integrated into NTS2. Arrows indicate direction of transcription; circles, ARS (origin of replication).

verify the presence and location of the *HIS3* gene in the rDNA locus in each strain, agarose-embedded chromosomal DNA was analyzed after digestion with a restriction enzyme by CHEF gel electrophoresis and Southern blotting. Integration of the *HIS3* gene introduces a single *Pst*I restriction site into the rDNA, allowing positional mapping (Figure 3-2A). Southern blots were analyzed by hybridization with a probe complementary to the 5' end of the *HIS3* gene. A second probe complementary to the NTS region of the rDNA revealed the sizes of the two rDNA-containing fragments (Figure 3-2B). By combining these two pieces of mapping data, the position of the *HIS3* gene within the rDNA array and the length of the rDNA array were determined. Digestion of genomic DNA with *Sfi*I, which cuts once in each repeat and once in the *HIS3* gene, followed by Southern blotting was used to verify that multimers of *HIS3* were not present in the rDNA (data not shown). Using these mapping techniques, the presence of a single reporter gene was verified. A collection of sixteen congenic yeast strains, each with a single *HIS3* reporter in the rDNA, was made, mapped (Figure 3-2C), and used to determine if the strength of silent chromatin in different rDNA repeat units is uniform.

3.3.2 Silencing of Pol II transcription is not the same in different repeats across the rDNA array

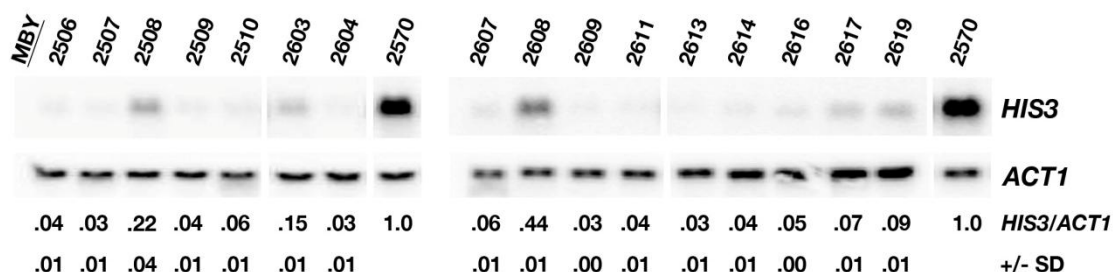
Transcription of Pol II genes integrated into the rDNA locus is repressed by silent chromatin formed by the actions of several proteins, including the RENT complex and Set1 [22, 26, 28, 35, 37]. To assess silent chromatin in different rDNA repeat units, the steady-state level of *HIS3* mRNA from sixteen yeast strains, each with a single *HIS3*

Figure 3-2. *HIS3* introduces a unique *Pst*I restriction site into the rDNA, allowing for positional mapping. (A) Schematic of *Pst*I restriction site introduced into the rDNA in the *HIS3* reporter gene. The restriction enzyme *Pst*I was used to digest agarose-embedded chromosomes to map the location of *HIS3* within the rDNA. (B) A CHEF gel Southern blot was first hybridized to a *HIS3* probe to determine the location of the *HIS3* gene. The blot was then hybridized to a probe specific for the rDNA NTS region to identify the two fragments containing the rDNA. H, *HIS3*-marked rDNA fragment; *, unmarked rDNA fragment. Undigested chr *XII* resolves as a doublet due to its large size (~1,845 kb). Three representative strains are shown with a single *HIS3* gene integrated in a different region of the rDNA array. CEN, *HIS3* at the centromere-proximal side of the rDNA array; MID, *HIS3* in the middle of the rDNA array; TEL, *HIS3* located at the telomere-proximal side of the array. (C) CHEF gel Southern blots of *Pst*I-digested agarose-embedded chromosomes hybridized to a *HIS3* probe to indicate the location of *HIS3* in the rDNA array. Sixteen NTS2::*HIS3* strains are shown.



gene integrated into NTS2 of an rDNA repeat, was measured by Northern blot. The ratio of *HIS3* mRNA/*ACT1* mRNA was determined for each strain, and then normalized to the *HIS3* mRNA/*ACT1* mRNA ratio from a yeast strain (MBY2570) with a *HIS3* gene integrated in a euchromatic region of chromosome (chr) *XII* outside the rDNA (chr*XII*::*HIS3*). Three independent experiments analyzing total RNA from the sixteen strains with *HIS3* in NTS2 (NTS2::*HIS3* strain collection) revealed that the level of *HIS3* mRNA was not the same in each strain. The normalized *HIS3*/*ACT1* mRNA levels among the sixteen NTS2::*HIS3* strains varied from 0.03 to 0.44 (Figure 3-3A), indicating that silent chromatin in different rDNA repeat units is not equally efficient at repressing Pol II transcription. In most of the NTS2::*HIS3* yeast strains analyzed, the normalized level of *HIS3* transcript was less than 10% (*HIS3*/*ACT1* mRNA ratio <0.10; Figure 3-3A) of the chr*XII*::*HIS3* control strain MBY2570. In three of the sixteen NTS2::*HIS3* strains, the normalized level of *HIS3* mRNA was 15% to 44% of the level in MBY2570. It is important to note that the value of the standard deviation for the *HIS3*/*ACT1* mRNA ratios across the three independent Northern blotting experiments is low (0.00-0.04). The low standard deviation value indicates that the steady-state level of mRNA from the *HIS3* gene in an individual strain is stable. This result suggests that the variation in the *HIS3*/*ACT1* mRNA levels among the sixteen yeast strains is due to differences in silent chromatin within individual rDNA repeat units. For these strains, no simple correlation was identified between the level of *HIS3* mRNA and the position of *HIS3* within the rDNA array; less silent repeats were found throughout the rDNA locus and not specifically closer to the centromere, middle, or telomere side of the rDNA array.

A.



B.

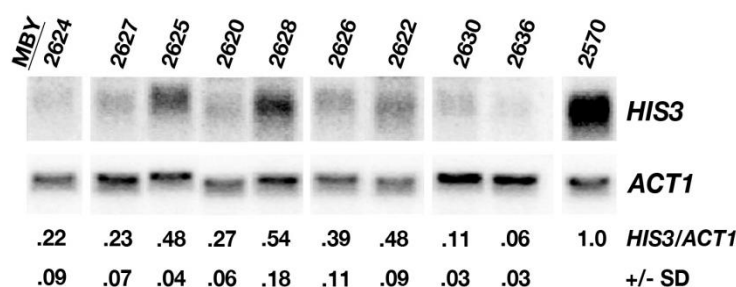


Figure 3-3. Pol II gene silencing of a *HIS3* reporter gene in different rDNA repeat units is not uniform. (A) Total RNA from sixteen yeast strains, each with a single *HIS3* gene in the NTS2 region of a single repeat unit in the rDNA array, was analyzed by Northern blot. *ACT1* mRNA levels were used to normalize loading. The average *HIS3/ACT1* mRNA ratio normalized to the *HIS3/ACT1* mRNA ratio of a control strain (MBY2570) with *HIS3* in a euchromatic region of chr *XII* and standard deviations (SD) were determined from three independent experiments. (B) Total RNA from nine strains having a single *HIS3* gene in NTS1 in different repeat units was analyzed by Northern blot as described in (A).

To investigate whether the variation observed in Pol II gene silencing (*HIS3* expression) is specific to NTS2, a second strain collection was made with each strain having a single *HIS3* gene integrated into the NTS1 region of the rDNA repeat (NTS1::*HIS3*). Northern analyses revealed that the normalized *HIS3/ACT1* mRNA levels among nine NTS1::*HIS3* strains varied from 0.06 to 0.54 (Figure 3-3B), indicating that silent chromatin in NTS1 is not uniform. The values of the standard deviation among the replicate experiments (Figure 3-3B) were low, indicating that the steady-state level of *HIS3* mRNA from the reporter integrated in the NTS1 region of a single rDNA repeat unit is stable. Silencing of the *HIS3* reporter gene was not as strong when integrated in the NTS1 region of the rDNA repeat relative to when it was located in NTS2 (compare normalized *HIS3/ACT1* mRNA levels in Figure 3-3A to those in 3-3B), consistent with a previous report that silent chromatin in NTS2 is more repressive than silent chromatin in NTS1 [22]. The observed variation in the degree of silencing conferred by rDNA chromatin is referred to as rDNA Position Effect (rDNA-PE).

In the NTS2::*HIS3* strain collection described above, the *HIS3* promoter is ~1 kb upstream of the 35S rRNA gene promoter and ~300 bp from the origin of DNA replication (ARS) (Figure 3-1); the direction of transcription of the *HIS3* reporter gene by Pol II is opposite that of transcription of the 35S rRNA gene by Pol I. To evaluate possible effects of the ARS and Pol I regulatory sequences on expression of the *HIS3* reporter gene, a strain collection was made with *HIS3* integrated at the same DNA sequence as the NTS2::*HIS3* strain collection but in the reverse orientation (NTS2::r*HIS3*). In this strain collection, the *HIS3* promoter is further away from the

ARS (~1.3 kb) and the 35S rRNA gene promoter (~2 kb), and Pol II transcription proceeds in the same direction as Pol I transcription. Steady-state *HIS3* mRNA was measured as in Figure 3-3. Consistent with the data shown in Figure 3-3, variation in the steady-state level of *HIS3* mRNA was observed when comparing the strains in the NTS2::*rHIS3* collection (Figure 3-4); normalized *HIS3/ACT1* mRNA values of the seven NTS2::*rHIS3* strains ranged from 0.06 to 0.30. These results suggest increasing the distance of the *HIS3* promoter from the Pol I-transcribed 35S rRNA gene and ARS, and the polarity of Pol II transcription of the *HIS3* reporter gene, do not abolish rDNA-PE.

To determine if rDNA-PE is specific to the *HIS3* gene, the *HIS3* reporter was replaced by homologous recombination with the *S. cerevisiae TRP1* gene in three of the NTS2::*HIS3* strains and the chrXII::*HIS3* control strain. Total RNA from the NTS2::*TRP1* and the chrXII::*TRP1* strains was analyzed by Northern (Figure 3-5). Variation among the *TRP1* mRNA levels from reporters in different rDNA repeat units was observed in the NTS2::*TRP1* strains, similar to what was seen with the collections of yeast strains with *HIS3* reporters in NTS2 or NTS1 (Figure 3-3). Interestingly, the *TRP1* replacement version of MBY2608, the NTS2::*HIS3* strain with the highest level of *HIS3* mRNA, had the highest ratio of *TRP1/ACT1* mRNA (Figure 3-5, MBY2856). In the other two NTS2::*TRP1* strains, MBY2855 and MBY2857, *TRP1* replaced the *HIS3* reporter in repeat units with stronger silencing of the *HIS3* reporter. The *TRP1* mRNA levels in these two NTS2::*TRP1* strains were lower than those in MBY2856 (Figure 3-5), consistent with the stronger silencing observed in the original NTS2::*HIS3* strains. In

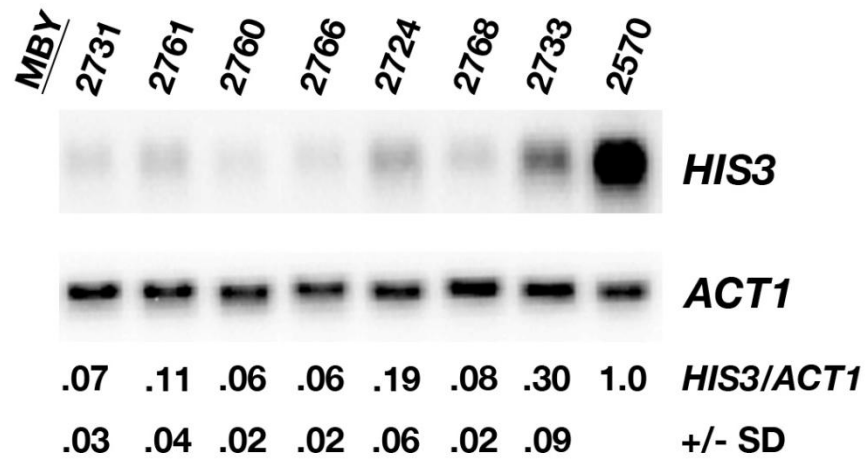


Figure 3-4. Changing the orientation of the *HIS3* gene in the rDNA does not abolish rDNA-PE. Total RNA from seven strains, each with a single *HIS3* gene in a different rDNA repeat unit (NTS2::r*HIS3* strain collection), was analyzed by Northern blot. Normalization and standard deviations were determined as in Figure 3-3A.

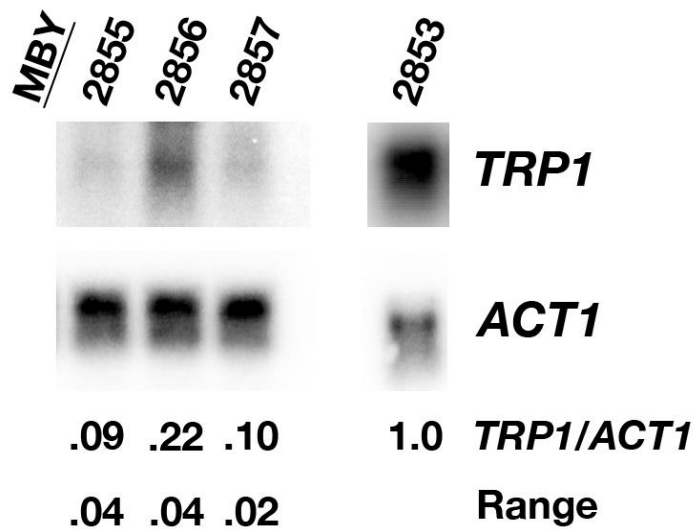


Figure 3-5. Variation in Pol II gene silencing is observed in strains with a *TRP1* reporter in the rDNA. A DNA fragment containing the *TRP1* gene was used to replace the *HIS3* gene in three NTS2::*HIS3* strains to make NTS2::*TRP1* strains. Total RNA (30 µg) from three strains with a *TRP1* gene in a different rDNA repeat unit was analyzed by Northern blot. *ACT1* mRNA levels were used to normalize loading. The average *TRP1/ACT1* RNA ratio was normalized to RNA (15 µg) from a control strain (MBY2853) with *TRP1* in a euchromatic region of chr *XII*. The average *TRP1/ACT1* mRNA ratio and range were calculated from two independent experiments.

summary, these data show that rDNA-PE can be detected using the *TRP1* gene, indicating the rDNA-PE is not specific to the *HIS3* gene.

3.3.3 Evidence that rDNA-PE affects the rate of mitotic recombination in the rDNA array

In addition to repressing Pol II transcription, silent chromatin in the rDNA represses mitotic recombination [29], thereby providing a mechanism to promote stability of the highly repeated rDNA locus [25, 40]. Recombination between rDNA repeats flanking the *HIS3*-marked repeat can cause loss of the *HIS3* reporter; the rate of loss of the *HIS3* gene from the rDNA during non-selective growth of a culture provides a measure of mitotic recombination in the rDNA. Recombination assays were performed on the sixteen NTS2::*HIS3* strains as well as the control chrXII::*HIS3* strain MBY2570. The rate of loss of the *HIS3* gene was calculated by comparing the number of His⁻ colonies relative to the total number of colonies from cultures plated after 120 generations of non-selective growth in rich media. The results show that the rate of loss of the *HIS3* gene from the rDNA per generation in the NTS2::*HIS3* strains varies from 0.6×10^{-4} to 10.0×10^{-4} (Table 3-3). The NTS2::*HIS3* strains lose the *HIS3* gene from the rDNA 2.7- to 41.8-fold more often than the control strain loses *HIS3* from chr XII (Table 3-3). These findings indicate that the silent chromatin in different rDNA repeat units is not uniform with respect to the ability to repress recombination among rDNA repeat units. The Pol II expression data and the mitotic recombination data from each of the sixteen NTS2::*HIS3* strains correlate well with each other (Figure 3-6). The findings suggest that the silent chromatin in some rDNA repeats is more permissive for Pol II

Table 3-3. Rate of loss of the *HIS3* gene from the rDNA.

MBY	Strain	<i>HIS3</i> loss +/- SD (x10⁻⁴) per generation	Rate of loss Relative to Control^a
2570	Control	0.2 +/- 0.24	-
2604	1	0.6 +/- 0.18	2.7
2611	2	0.8 +/- 0.21	3.4
2507	3	0.9 +/- 0.19	3.7
2609	4	1.1 +/- 0.25	4.6
2607	5	1.1 +/- 0.32	4.6
2614	6	1.4 +/- 0.74	5.8
2613	7	1.4 +/- 0.25	5.8
2506	8	1.6 +/- 0.49	6.8
2619	9	1.7 +/- 0.23	7.0
2616	10	1.8 +/- 0.51	7.4
2509	11	1.8 +/- 0.44	7.7
2617	12	2.9 +/- 0.43	12.2
2510	13	3.0 +/- 0.35	12.3
2508	14	3.4 +/- 0.48	14.2
2603	15	3.5 +/- 1.0	14.6
2608	16	10.0 +/- 0.89	41.8

^a Control, yeast strain MBY2570 has the *HIS3* gene integrated into a euchromatic portion of chr *XII*.

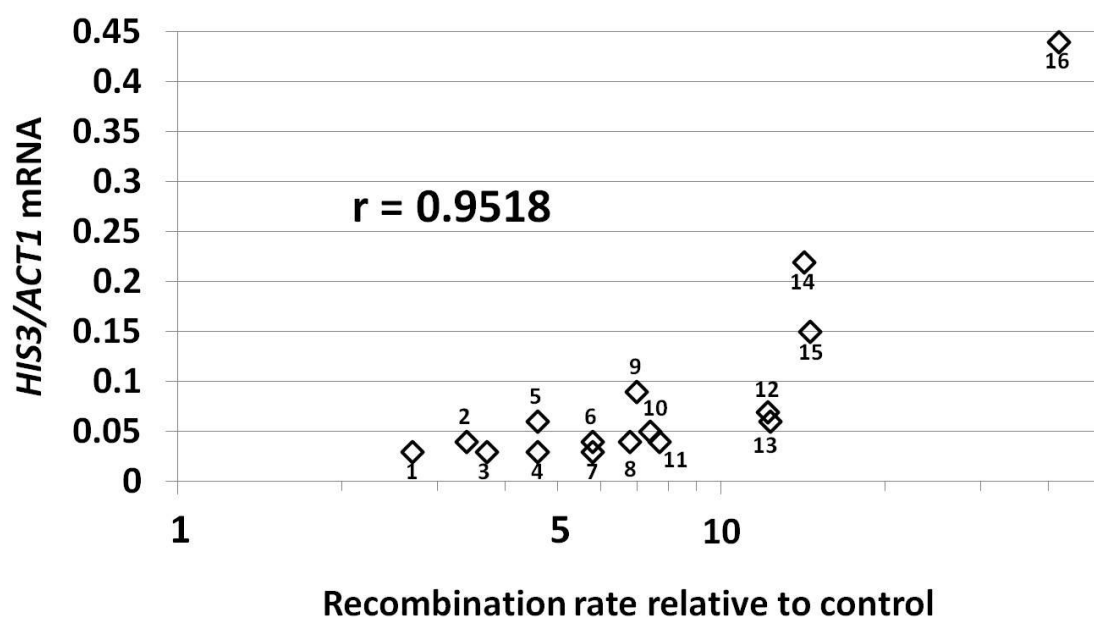


Figure 3-6. Two properties of silent chromatin, Pol II gene silencing (y-axis) and mitotic recombination (x-axis), correlate in the sixteen *NTS2::HIS3* strains. The data indicate that rDNA repeat units with stronger transcriptional silencing have lower rates of mitotic recombination. The relationship suggests that rDNA-PE is a property of rDNA silent chromatin that affects two of its known functions.

transcription and recombination; in other rDNA repeats the silent chromatin is more repressive as reflected by lower levels of Pol II transcription and mitotic recombination. Altogether, these results support the hypothesis that silent chromatin is not equivalent in different rDNA repeat units.

3.3.4 Sequence differences are not a factor in rDNA-PE

To rule out that a change in the sequence of the *HIS3* reporter gene is the cause for the differences in *HIS3* transcript levels the promoter and first ~200bp of the *HIS3* open reading frame were sequenced in all sixteen NTS2::*HIS3* strains. In fourteen of the sixteen strains, the *HIS3* reporter gene and associated sequences had the consensus DNA sequence. Two of the sixteen strains were found to have base substitution mutations upstream of *HIS3*. MBY2608 has a T to G substitution 24 bases upstream of the *HIS3* start codon. To determine if this substitution altered expression, the allele was introduced into a euchromatic region of chr *XII* in a *his3Δ200* strain. Northern blotting experiments showed that transcript level from the allele with the T-G substitution was similar to that from a wild-type *HIS3* gene in the same position (wild-type *HIS3/ACT1* mRNA ratio = 1.0; T-G substitution normalized *HIS3/ACT1* mRNA ratio = 1.06 +/- 0.05). In strain MBY2617, a C to T substitution was detected 187 bases upstream of *HIS3* start codon. This substitution is in a region upstream of *HIS3* that can be deleted without any affect on *HIS3* expression [139]. In addition, DNA sequences 500 bases upstream and 500 bases downstream of the *HIS3* insert in each marked rDNA repeat unit were verified in five strains and no sequence differences were found (data not shown).

These data suggest that rDNA-PE is not caused by sequence differences in the *HIS3* reporter gene or in nearby rDNA sequences.

3.3.5 rDNA-PE in cells lacking the histone methyltransferase Set1

Set1, the histone methyltransferase responsible for methylation of lysine 4 in the tail of histone H3 in *S. cerevisiae*, has been shown to play a role in silencing Pol II-transcribed genes in the rDNA [35, 41]. Loss of Set1 function results in a two- to three-fold reduction in Pol II gene silencing at the rDNA, but has little or no effect on the rate of mitotic homologous recombination in the rDNA [35]. To determine if Set1 or K4-methylation of histone H3 regulates rDNA-PE, the *SET1* gene was deleted and replaced with *KANMX4* in five NTS2::*HIS3* strains. Northern analyses with these strains showed that the normalized *HIS3/ACT1* mRNA levels among the seven *set1*Δ NTS2::*HIS3* strains varied from 0.12 to 0.37 (Figure 3-7). If Set1 was the sole protein required for rDNA-PE, then the *HIS3* gene in each *set1*Δ strain should be expressed at the same level, with no variation in steady state *HIS3* transcript among *set1*Δ strains. However, the data show variation in the degree of Pol II transcriptional silencing occurs in the absence of Set1, indicating Set1 activity is not required for rDNA-PE.

In four of the seven NTS2::*HIS3 set1*Δ strains, the *HIS3*-marked repeat is in the same location within the rDNA array as the *SET1*⁺ parent, therefore the fold-change in *HIS3* transcript in *set1*Δ compared to *SET1*⁺ strains can be calculated in these four strains. Consistent with what has been observed previously [35], total mRNA from three of the four *set1*Δ strains showed an increase in *HIS3* mRNA of 1.47 to 2.57-fold compared to the respective *SET1*⁺ parent strain (Table 3-4). In contrast, MBY2962, a

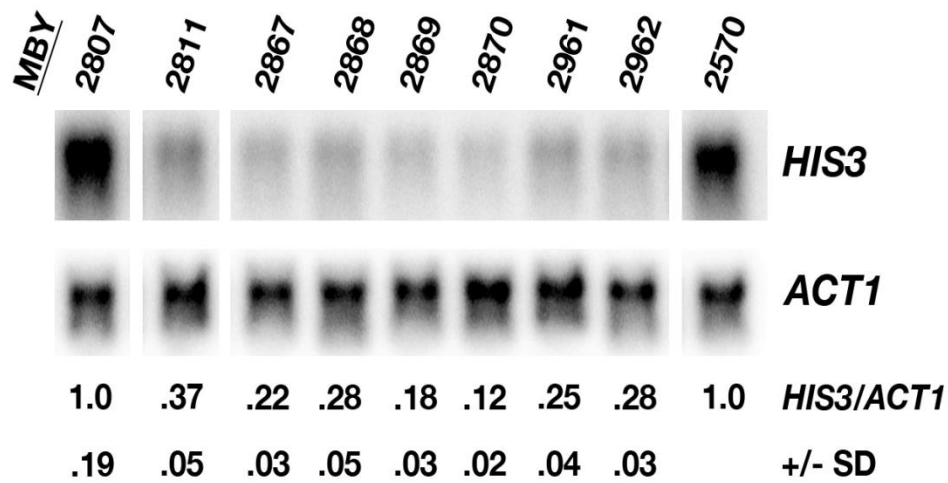


Figure 3-7. Strains lacking Set1 exhibit variable levels of *HIS3* transcript. Total RNA from seven NTS2::*HIS3 set1Δ* strains was analyzed by Northern blot. *ACT1* RNA serves as a loading control. MBY2807 is *set1Δ* with *HIS3* in a euchromatic region of chr *XII* and is included as a control, showing the loss of Set1 does not affect *HIS3* transcription outside of the rDNA (compare *HIS3/ACT1* ratio to MBY2570). Normalization and calculation of standard deviations was performed as in Figure 3-3A.

Table 3-4. Comparison of *HIS3* expression in *SET1*⁺ vs. *set1Δ* cells.

MBY (<i>SET1</i>⁺)	<i>HIS3/ACT1</i> mRNA	MBY (<i>set1Δ</i>)	<i>HIS3/ACT1</i> mRNA	Fold Change^a <i>set1Δ/SET1</i>⁺
2508	0.22	2811	0.37	---
2603	0.15	2867	0.22	1.47
		2868	0.28	1.87
2617	0.07	2869	0.18	2.57
		2870	0.12	---
2608	0.44	2961	0.25	---
		2962	0.28	0.64

^a Fold change calculated from *SET1*⁺/*set1Δ* strain pairs with similar sized fragments detected in CHEF gel Southern blots used to map the location of the *HIS3* reporter within the rDNA array. Fragment sizes within mapping error of less than six rDNA repeat units are considered to be the same size (see Materials and Methods, Section 3.2.3).

set1Δ isolate of the NTS2::*HIS3* yeast strain MBY2608, expression of the *HIS3* gene was decreased, resulting in a fold-change of 0.64 in the *set1Δ* strain compared to the *SET1*⁺ parent. This observation is intriguing because no data previously published has shown an increase in rDNA silencing following the loss of H3K4 methylation. Variation in the fold-change in *HIS3* expression is additional supporting data that Set1 is not essential for rDNA-PE.

3.3.6 The levels of Sir2 protein in whole cell extracts in congenic yeast strains are similar

The NAD⁺-dependent histone deacetylase Sir2, a member of the RENT complex, is required for silencing of Pol II transcription and mitotic recombination at the rDNA [26, 28-30, 37]. Previous work has shown that Sir2 is limiting for silent chromatin at the rDNA; when Sir2 is overexpressed, silencing of Pol II genes in the rDNA becomes stronger [36]. One possibility to explain rDNA-PE is that different levels of Sir2 protein are present in the yeast strains used in this study. A strain with more Sir2 would be expected to have more repressive rDNA silent chromatin, therefore lower levels of *HIS3* transcript, while a strain with less Sir2 is expected to have less repressive rDNA silent chromatin, therefore higher levels of *HIS3* transcript. Differences in the level of Sir2 protein in these strains are unexpected because the strains are congenic; the only known difference is the position of the *HIS3*-marked rDNA repeat unit within the rDNA array.

To determine if cellular levels of Sir2 are similar in strains used in this study, Western analysis was performed on whole cell protein extracts from four NTS2::*HIS3* strains and the control strain *chrXII*::*HIS3*. The results show that the level of Sir2

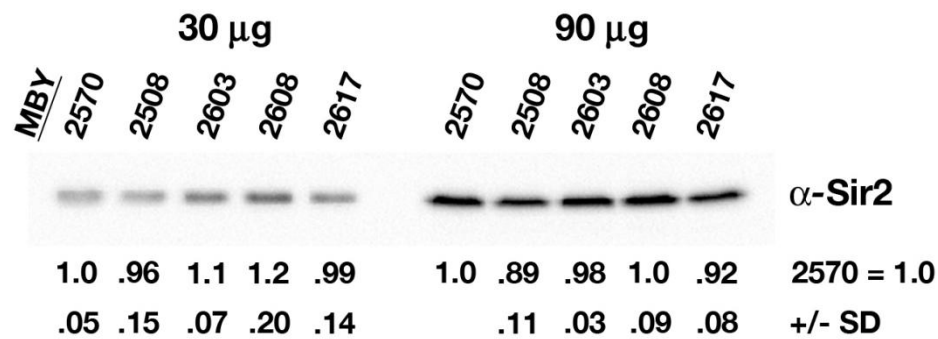


Figure 3-8. Levels of Sir2 protein in whole cell extracts are similar in four NTS2::*HIS3* strains and MBY2570 (*chrXII::HIS3*). Whole cell extracts (30 µg and 90 µg) were separated on SDS-PAGE and transferred to PVDF membrane. The membrane was probed with a Sir2-specific antibody. A representative Western blot is shown. Signal from each sample was normalized to MBY2570 (90 µg) for comparison and standard deviations (SD) were determined from three independent experiments.

protein was similar in the yeast strains analyzed (Figure 3-8), indicating that rDNA-PE is unlikely to be due to differences in cellular Sir2 protein levels.

3.3.7 Sir2 association with the *HIS3*-marked repeat is variable

It is possible that rDNA-PE is the result of different levels of Sir2 associating with different *HIS3*-marked rDNA repeat units. Chromatin immunoprecipitation (ChIP) was performed to measure the association of Sir2 protein with the *HIS3*-marked rDNA repeat using yeast strains from the NTS2::*HIS3* and NTS1::*HIS3* collections. The expectation is that the level of Sir2 associated with the *HIS3*-marked repeat will correlate inversely with the strength of silencing; Sir2 association will be lower in less silent rDNA repeats compared to the more silent rDNA repeats.

DNA from chromatin immunoprecipitated with Sir2 antisera was purified and analyzed by quantitative PCR (qPCR). Two primer sets were used, one to assess the association of Sir2 with the *HIS3*-marked rDNA repeat unit, and a second set to measure the association of Sir2 with unmarked rDNA repeat units. The %IP of Sir2 at the *HIS3*-marked rDNA repeat was normalized to the %IP of Sir2 in all unmarked rDNA repeats (Figure 3-9A). ChIP experiments revealed that yeast strain MBY2617, which exhibited strong silencing of the *HIS3* gene in the rDNA (Figure 3-3A, *HIS3/ACT1* mRNA = 0.07), had significantly more Sir2 associated with the *HIS3*-marked rDNA repeat than two of the other NTS2::*HIS3* strains, MBY2603 and MBY2608 (Figure 3-9A). MBY2617 also had higher average Sir2 association with the *HIS3*-marked repeat than MBY2508, but the numbers were not statistically significant. Similar levels of transcript from the *HIS3* gene in the rDNA were detected in yeast strains MBY2508 and

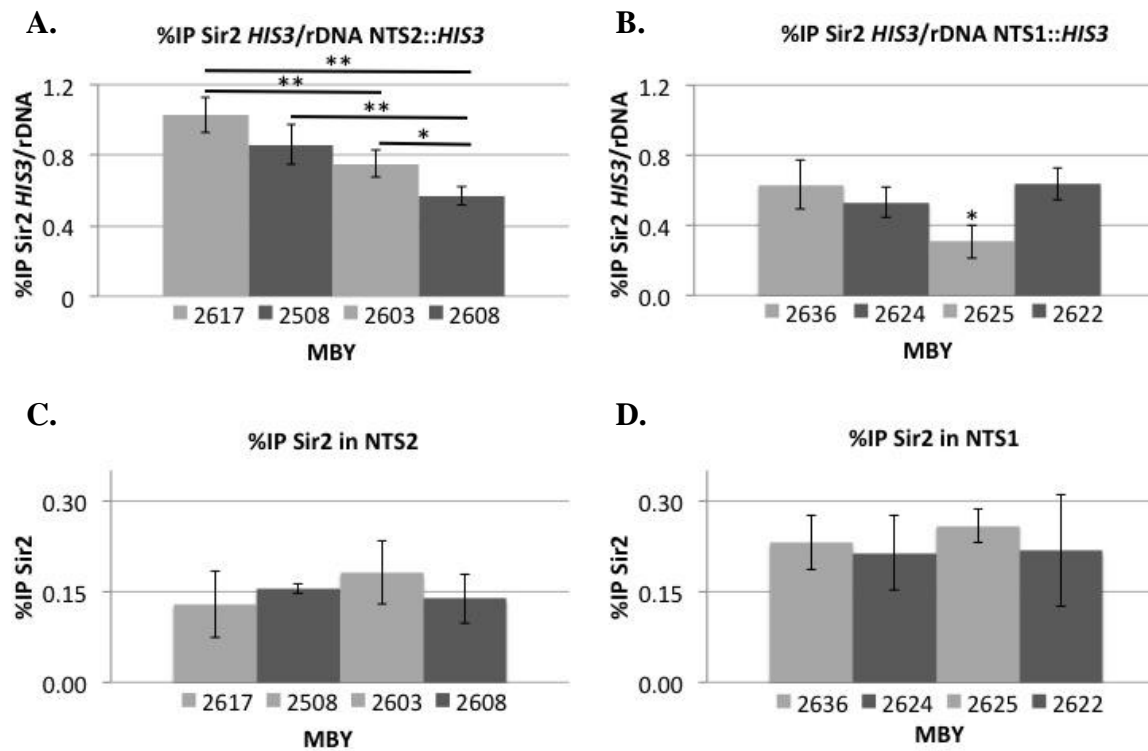


Figure 3-9. Chromatin immunoprecipitation (ChIP) with Sir2 antibody shows association of Sir2 with different *HIS3*-marked or unmarked rDNA repeats. (A) %IP of Sir2 with the *HIS3* gene in yeast strains from the NTS2::*HIS3* collection. (B) %IP of Sir2 with the *HIS3* gene in yeast strains from the NTS1::*HIS3* collection. (C) %IP of Sir2 with NTS2 in yeast strains from the NTS2::*HIS3* collection. (D) %IP of Sir2 with NTS1 in yeast strains from the NTS1::*HIS3* collection. Yeast strain numbers are shown on the X axis. %IP values are shown on the Y axis. The %IP Sir2 with the *HIS3* reporter in NTS2 (A) or NTS1 (B) was normalized to %IP Sir2 with NTS2 (C) or NTS1 (D) of unmarked rDNA repeats. * $p < 0.05$; ** $p < 0.01$.

MBY2603 (Figure 3-3A, *HIS3/ACT1* mRNA ratios of 0.22 and 0.15, respectively), and Sir2 association with the *HIS3*-marked repeat in these strains was similar (Figure 3-9A). Consistent with this trend, yeast strain MBY2608, that had the highest level of transcript from the *HIS3* gene in the rDNA (Figure 3-3A, *HIS3/ACT1* mRNA ratio = 0.44) had significantly less Sir2 associated with the *HIS3*-marked rDNA repeat than the three other NTS2::*HIS3* strains tested (Figure 3-9A). These data support the hypothesis that the level of Sir2 associated with the *HIS3*-marked rDNA repeat correlates with the strength of Pol II gene silencing in NTS2; more Sir2 was associated with the most silent *HIS3*-marked rDNA repeat unit and less Sir2 was associated with the least silent *HIS3*-marked rDNA repeat unit.

The association of Sir2 with *HIS3*-marked rDNA repeats was analyzed in four NTS1::*HIS3* strains using ChIP and qPCR as described above. MBY2625, one of the least silent strains in the NTS1::*HIS3* strain collection (Figure 3-3B, *HIS3/ACT1* mRNA ratio = 0.48) had significantly less Sir2 associated with the *HIS3*-marked repeat than two strains in which silencing of *HIS3* was stronger, MBY2636 and MBY2624 (Figure 3-3B, *HIS3/ACT1* mRNA ratio = 0.06 and 0.22, respectively) (Figure 3-9B). These results corroborate the observations from the NTS2::*HIS3* strains (Figure 3-9A) and suggest that less Sir2 is associated with the *HIS3*-marked rDNA repeats that are more highly expressed.

While these data support the hypothesis that Sir2 association is lower with less silent *HIS3*-marked rDNA repeats, some strains in the NTS1::*HIS3* collection had results that were inconsistent with this simple hypothesis. For example, Sir2 association with

the *HIS3*-marked rDNA repeat in yeast strain MBY2636 was not significantly different from that in MBY2624, despite the difference in normalized *HIS3* transcript levels (Figure 3-3B, 0.06 and 0.22, respectively). Further, in MBY2622, the steady state level of *HIS3* mRNA was identical to that in yeast strain MBY2625 (Figure 3-3B, *HIS3/ACT1* mRNA, 0.48); based on the simple hypothesis, MBY2622 was expected to have a lower level of Sir2 associated with the *HIS3*-marked repeat, similar to the level of association of Sir2 with *HIS3* in yeast strain MBY2625. However, MBY2622 had significantly more Sir2 associated with the *HIS3*-marked repeat than MBY2625, with a level of Sir2 association similar to strains MBY2636 and MBY2624 that have more silent *HIS3*-marked rDNA repeats. These contrary data suggest that Sir2 association with *HIS3*-marked repeats in NTS1 is not the sole determinant of the strength of silent chromatin. It is possible that other proteins or differences in chromatin structure specific to NTS1 may be involved in determining the strength of silent chromatin.

The association of Sir2 with the NTS2 and NTS1 regions of the unmarked rDNA repeats was also analyzed by qPCR (Figure 3-9 C&D). No differences were found in the level of association of Sir2 with unmarked rDNA repeats, consistent with our results showing that the level of Sir2 protein in whole cell extracts from these strains was similar (Figure 3-8). These data indicate that variation in the level of Sir2 detected by ChIP is specific to the *HIS3*-marked repeat and not a reflection of variation in the average association of Sir2 across the entire rDNA array.

3.3.8 The level of acetylated histone H3 at the *HIS3*-marked repeat is lower in the strain with the highest Sir2 association

Sir2 is an NAD⁺-dependent histone deacetylase, therefore it is expected that the level of acetylated histone H3 (AcH3) would correlate inversely with the association of Sir2 with a *HIS3*-marked rDNA repeat. Further, higher levels of acetylated histones are expected at the *HIS3*-marked repeats that are more highly expressed (less silent) [140]. ChIP experiments using antibody specific for AcH3 were performed with three NTS2::*HIS3* strains to examine the level of K9- and K14-AcH3 at the *HIS3*-marked rDNA repeat unit and the unmarked rDNA repeat units. The immunoprecipitated chromatin was purified and the resulting DNA was analyzed by qPCR.

The level of AcH3 at the *HIS3*-marked repeat in MY2617 was significantly lower than that in MBY2508 and MBY2608 (Figure 3-10A). This result is consistent with Sir2 ChIP data showing that MBY2617 has more Sir2 associated with the *HIS3*-marked repeat (Figure 3-9A). No difference in the level of AcH3 at the *HIS3*-marked repeats in MBY2508 and MBY2608 (Figure 3-10A) was detected; this was unexpected because Sir2 ChIP experiments showed MBY2508 had significantly more Sir2 at the *HIS3*-marked repeat than MBY2608 (Figure 3-9A) and Northern analyses showed a two-fold difference in steady state *HIS3* mRNA levels between the two strains (Figure 3-3A). A correlation between active Pol II transcription and acetylated histone H3 has been established [141]. It is possible that there is a threshold effect such that this correlation fails to hold true for Pol II genes located in the rDNA that are expressed at low levels. No significant differences in average AcH3 levels were detected in the NTS2 regions of

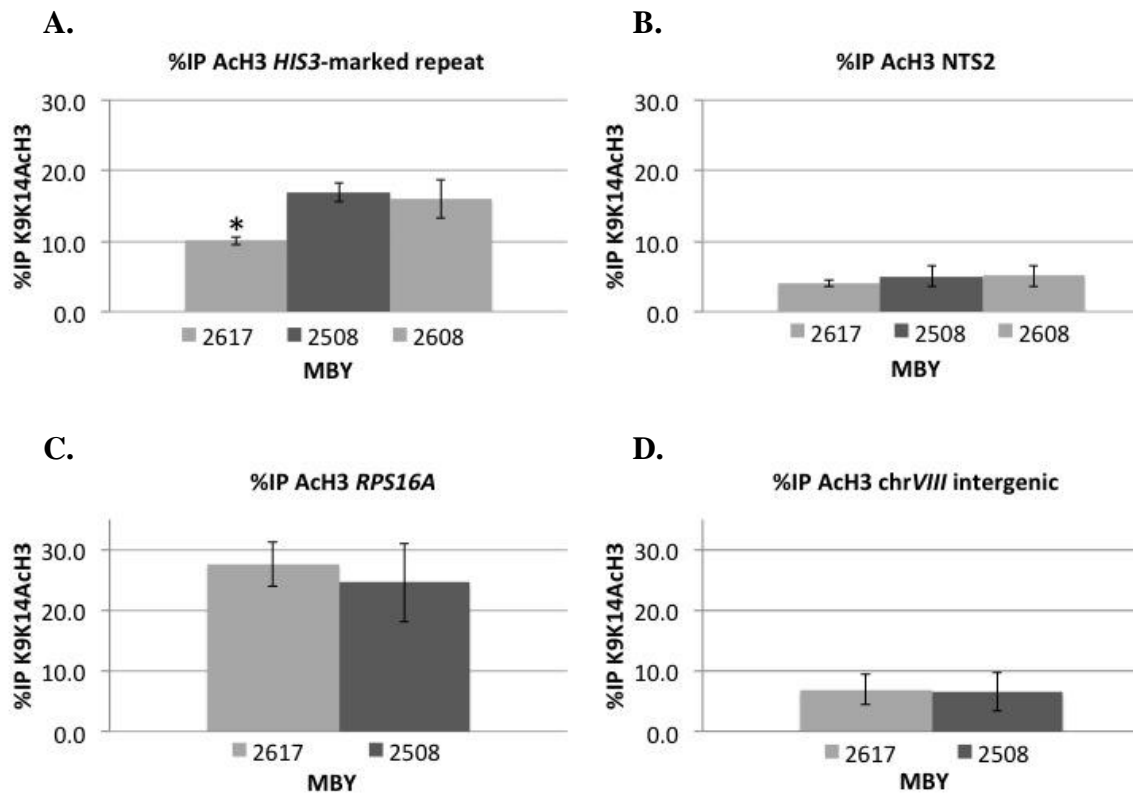


Figure 3-10. ChIP for K9-, K14-acetylated histone H3 in the rDNA NTS2 and *HIS3*-marked rDNA repeat in three strains. (A) %IP AcH3 in *HIS3*-marked repeat of three NTS2::*HIS3* strains. MBY2617 has significantly less AcH3 than the other two strains, consistent with the findings that there is more Sir2 associated with this *HIS3*-marked rDNA repeat in this yeast strain. (B) %IP AcH3 in the unmarked NTS2 regions of three of the NTS2::*HIS3* strains is similar. (C) *RPS16A*, a highly expressed gene, is used as a control and has high levels of AcH3. (D) Intergenic chr *XII* is a control for low %IP of AcH3. * $p < 0.05$.

unmarked rDNA repeat units in the strains analyzed (Figure 3-10B). This result is consistent with the Sir2 ChIP data showing that similar levels of Sir2 associated with the NTS2 regions of the rDNA (Figure 3-9C).

Quantitative PCR was performed to assess %IP AcH3 at two control loci outside the rDNA. No difference in AcH3 at either control locus was expected. *RPS16A*, a highly expressed Pol II-transcribed gene expected to have a high level of acetylated histones, had an average %IP of 24.6% in MBY2508 and 27.7% in MBY2617 (Figure 3-10C). An intergenic region of chr *VIII*, expected to have low levels of acetylated histones, had an average %IP of 6.5% in MBY2508 and 6.8% in MBY2617 (Figure 3-10D). There were no statistical differences in %IP at either locus between the two strains.

3.3.9 Evidence for Sir2-independent memory of the strength of silent chromatin at the rDNA

Sir2 appears to be a primary factor determining the strength of silent chromatin in NTS2 of individual rDNA repeat units. The mechanism behind how chromatin structure and Sir2 association is different in individual rDNA repeats that have the same sequence is unknown. Because silent chromatin and association of Sir2 can be different among individual rDNA repeat units, it is possible that different rDNA repeat units would have a different response to changes in the level of Sir2 in the cell. The question was addressed: following the loss and re-accumulation of Sir2, does silent chromatin in an rDNA repeat unit maintain the same strength of silencing it had prior to the loss of Sir2, or is new silent chromatin established with different strength of silencing?

Knowledge of how silent chromatin is established after the loss and re-accumulation of Sir2 could shed light on the role of Sir2 in rDNA-PE.

Excess hyper-recombination between rDNA repeats in the absence of Sir2 leads to movement or loss of the *HIS3*-marked rDNA repeat, therefore using a collection of NTS2::*HIS3 sir2Δ* strains is not a viable option to study a single rDNA repeat unit (R.A.J. unpublished data). In order to study silent chromatin in a single *HIS3*-marked rDNA repeat that remains in the same position within the rDNA array, a Tet-off system was employed to regulate the expression of Sir2. This system allows for the depletion and subsequent re-expression of Sir2 to occur quickly so that the *HIS3*-marked rDNA repeat is not moved or lost due to hyper-recombination while Sir2 is absent from the cells.

In this Tet-off system the endogenous *SIR2* promoter is replaced with two copies of the bacterial Tn10 *tet* operator and the TATA sequences of the *CYC1* promoter (*tetO₂-SIR2*); transcription of *SIR2* from this regulatory promoter is dictated by a tetracycline transactivator (tTA) containing the activation domain of a weak activator, Gcn4A, fused to the tetracycline repressor DNA binding domain [125]. During growth in the absence of a tetracycline analog, doxycycline, the tTA binds to *tetO₂-SIR2* and drives transcription of *SIR2*. Upon the addition of doxycycline to growth media, the tTA preferentially binds doxycycline, removing the activator from *tetO₂-SIR2* proximity and stopping *SIR2* transcription; rDNA silent chromatin is compromised upon Sir2 depletion. After removal of doxycycline by washing cells with sterile water and growth on fresh solid media without doxycycline, *SIR2* transcription begins and Sir2 accumulates in the

cell, thus re-establishing rDNA silent chromatin. Using this Tet-off system, the strength of rDNA silent chromatin in a single rDNA repeat unit can be assessed in cells either treated or not treated with doxycycline (Figure 3-11).

Duplicate cultures in rich media were inoculated from a single saturated culture; doxycycline (10 µg/ml) was added to one of the cultures (+ Dox). After six hours of growth, to allow for depletion of Sir2 in the culture containing doxycycline, cells from both cultures were washed with sterile water to remove doxycycline from the outside of the cells and single colony purified on rich solid media to allow for re-accumulation of Sir2 in the + Dox culture. The No Dox culture was included as a control to determine how much variability in silent chromatin occurs when the cells are grown without doxycycline. rDNA silent chromatin was analyzed in multiple colonies from both + Dox and No Dox cultures after recovery (Figure 3-11). Comparison of rDNA silent chromatin in colonies arising from cultures not treated with doxycycline to those treated with doxycycline will reveal whether the strength of silent chromatin in a single rDNA repeat unit is different after Sir2 has been lost and re-gained compared to cells that did not lose Sir2.

In most experiments, depletion and recovery of Sir2 was verified by Western blot; Sir2 was depleted after six hours growth with doxycycline and Sir2 returned to similar levels in each colony after recovery growth (data not shown). In most experiments whole chromosomes in agarose plugs were made from the starting saturated culture and from each colony to assess movement of the *HIS3*-marked repeat after the loss and recovery of Sir2. *Pst*I-digested chromosomes were analyzed by CHEF-gel

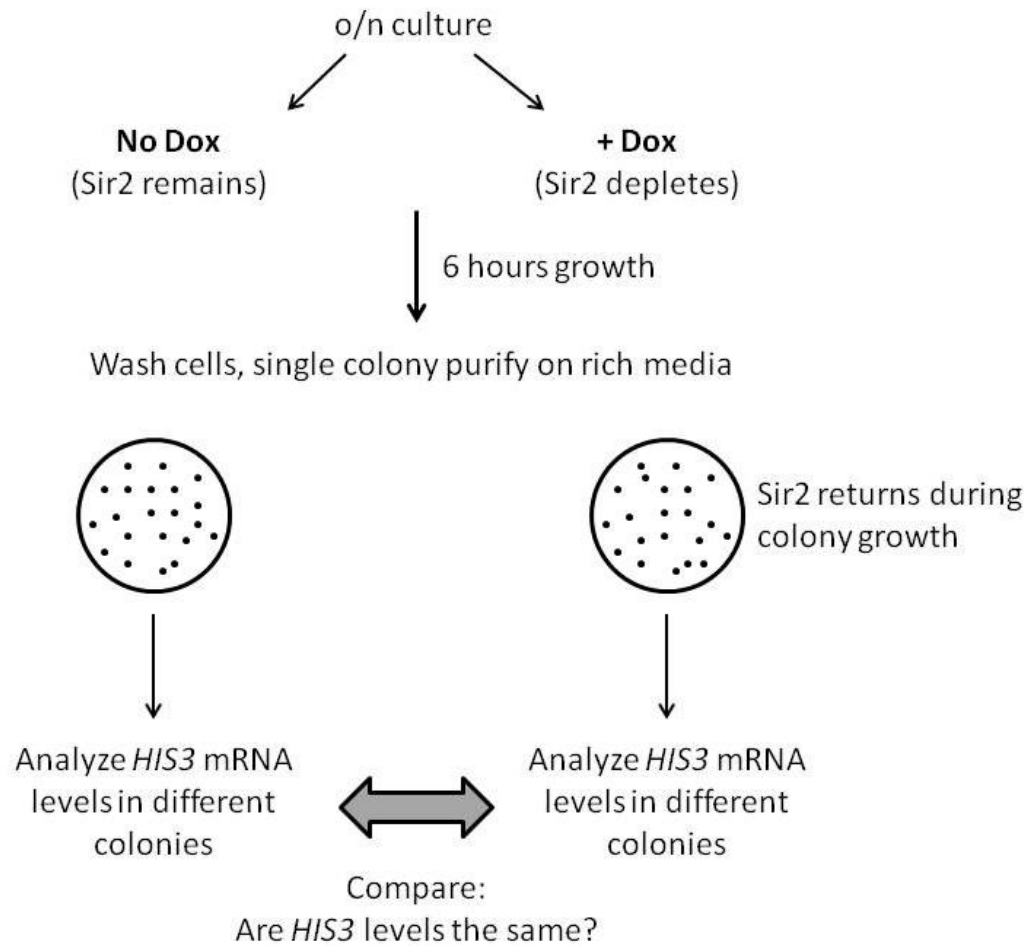


Figure 3-11. Diagram of Tet-Off single colony comparison experiments.

Southern blot as described in Figure 3-2 and little to no movement of the *HIS3*-marked repeat in the rDNA was detected; when changes were detected it was in the form of multiple *HIS3* inserts (data not shown). *HIS3* expression in colonies with multiple *HIS3* inserts in the rDNA resulted in transcript levels close to the euchromatic control (MBY2570), and these colonies were removed from the analysis as they are not a reflection of silent chromatin within a single *HIS3*-marked rDNA repeat.

RNA from colonies was analyzed by Northern blot for *HIS3* transcript levels as in Figure 3-3. A representative Northern blot of one experiment is shown in Figure 3-12. After six hours growth with doxycycline silent chromatin is compromised, as indicated by the 6.5-fold increase in *HIS3* transcript (Figure 3-12, lane 2) compared to the culture grown six hours without doxycycline (Figure 3-12, lane 1). Following recovery growth on rich media without doxycycline two colonies were analyzed from the No Dox culture (Figure 3-12, lanes 3 and 4) and had normalized *HIS3/ACT1* mRNA ratios of 0.25 and 0.22. Four colonies arising from the culture grown with doxycycline were analyzed (Figure 3-12, lanes 5-8). The *HIS3/ACT1* mRNA ratio of these four colonies ranged from 0.15 to 0.20.

This experiment was repeated at least twice with four Tet-off strains. Table 3-5 contains a summary of results from the four strains analyzed after single colony purification following growth with or without doxycycline. Depletion of Sir2 by growth with doxycycline was repeated at least twice with each of the four yeast strains.

After six hours growth with doxycycline, the *HIS3/ACT1* mRNA ratio in each strain was close to or exceeded that of the euchromatic control (*HIS3/ACT1* mRNA =

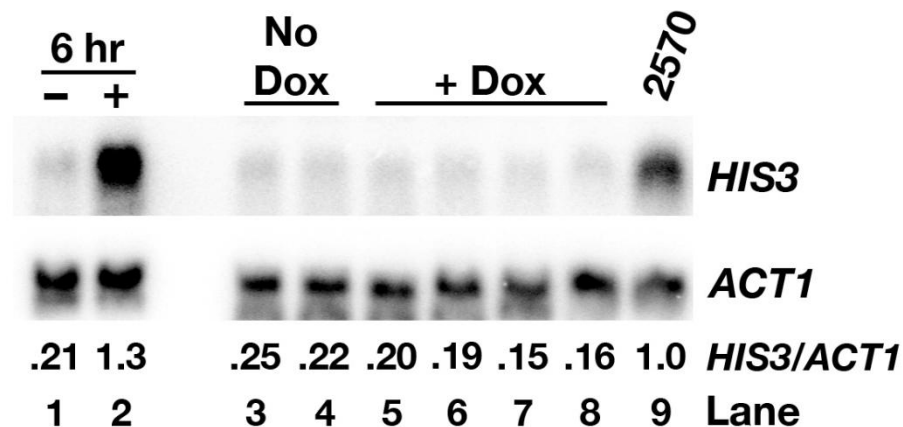


Figure 3-12. The strength of rDNA silent chromatin remains the same after the loss and re-accumulation of Sir2. A Tet-Off system was used to control *SIR2* expression in yeast strain MBY2948. After six hours growth in the presence of 10 μ g/ml doxycycline Sir2 has depleted and rDNA silent chromatin is compromised, as indicated by the increase in *HIS3* expression (lane 2). Following the removal of doxycycline from the + Dox culture and growth on rich media, *HIS3* levels from colonies arising from the No Dox (lanes 3 and 4) and + Dox (lanes 5-8) cultures was analyzed by Northern blot. No statistical differences in average *HIS3* expression were found between No Dox or + Dox colonies compared to the *HIS3* expression of the six hour No Dox sample (lane 1). Normalization was preformed as in Figure 3-3A.

Table 3-5. Analysis of silent chromatin in single colonies following depletion and accumulation of Sir2.

MBY2851	# colonies	<i>HIS3/ACT1</i> mRNA	Range or +/-SD
6 hour No Dox		0.444	0.077
6 hour + Dox		0.795	0.212
No Dox colonies	2	0.278	0.018
+ Dox colonies	6	0.241	0.030
P-value = 0.060			

MBY2948	# colonies	<i>HIS3/ACT1</i> mRNA	Range or +/-SD
6 hour No Dox		0.210	0.096
6 hour + Dox		1.038	0.280
No Dox colonies	7	0.182	0.048
+ Dox colonies	15	0.180	0.059
P-value = 0.941			

MBY2951	# colonies	<i>HIS3/ACT1</i> mRNA	Range or +/-SD
6 hour No Dox		0.324	0.101
6 hour + Dox		1.547	0.587
No Dox colonies	6	0.361	0.109
+ Dox colonies	13	0.331	0.055
P-value = 0.539			

MBY2954	# colonies	<i>HIS3/ACT1</i> mRNA	Range or +/-SD
6 hour No Dox		0.295	n/a
6 hour + Dox		1.377	0.914
No Dox colonies	4	0.241	0.055
+ Dox colonies	7	0.305	0.125
P-value = 0.270			

1.0), indicating that loss of Sir2 resulted in a loss of rDNA silent chromatin (Table 3-5, rows “6 hours + Dox”). After six hours growth without doxycycline (Table 3-5, rows “6 hours No Dox”) there is less *HIS3* transcript than the culture grown with doxycycline, indicating rDNA silent chromatin was retained in the No Dox culture because Sir2 was not depleted by doxycycline.

HIS3/ACT1 mRNA ratio of each colony was pooled to determine the average *HIS3/ACT1* mRNA ratio in No Dox colonies and + Dox colonies (Table 3-5). The total number of colonies analyzed for each yeast strain in each growth condition is specified in Table 3-5. Low standard deviations were calculated for each strain in both No Dox and + Dox colonies, indicating that the level of *HIS3* transcript from different colonies of the same strain was comparable.

The pooled *HIS3/ACT1* mRNA ratios of No Dox colonies were compared by T-test to the pooled *HIS3/ACT1* mRNA ratios of + Dox colonies to determine if there was a difference in the level of *HIS3* transcript in cells that lost Sir2. No statistically significant difference in *HIS3/ACT1* mRNA ratios was found (Table 3-5, p-values>0.05). A p-value of 0.06 was calculated for MBY2851 No Dox colonies compared to + Dox colonies; while not a significant difference, this p-value was lower than the p-value calculated from the other three yeast strains, likely due to the comparatively few number of colonies analyzed.

No difference in *HIS3/ACT1* mRNA ratios in No Dox colonies compared to + Dox colonies suggests that rDNA repeats have a Sir2-independent mechanism to “remember” the strength of silencing within an rDNA repeat unit such that upon the

return of Sir2 silent chromatin is re-established to the same strength as in cells that did not lose Sir2. The four strains presented here have normalized *HIS3/ACT1* mRNA ratios between 0.18 to 0.36 (Table 3-5). Analysis of additional strains with stronger rDNA silencing (lower *HIS3/ACT1* mRNA ratio) would provide additional insight into the regulation of rDNA-PE by Sir2.

3.4 DISCUSSION

3.4.1 Silent chromatin is not equal across the rDNA array

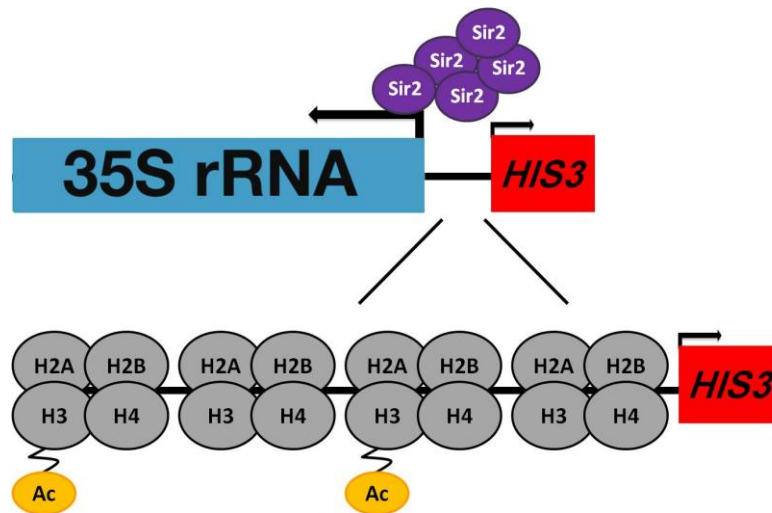
Silent chromatin at the rDNA locus contributes to genome stability by repressing Pol II transcription in the rDNA and homologous recombination between rDNA repeats. Silent chromatin at the rDNA has been assumed to be uniform across the approximately 200 rDNA repeat units that comprise the rDNA array, but until now this has not been tested. To analyze silent chromatin in individual rDNA repeat units, a *HIS3* reporter gene was integrated into the same primary DNA sequence in different rDNA repeat units across the rDNA array to make congenic collections of strains. Pol II transcription and mitotic recombination, two processes repressed by silent chromatin, were analyzed in yeast strains that each have a different rDNA repeat unit marked with a *HIS3* reporter gene. Functional assays revealed that silent chromatin in these different rDNA repeat units is not uniform; Pol II transcription and mitotic recombination in the rDNA can vary in a position-dependent manner, yet the strength of silent chromatin within a single rDNA repeat unit is stable. Variation in silent chromatin in individual rDNA repeat units is a property now referred to as rDNA Position Effect (rDNA-PE).

Northern blot analyses and ChIP experiments were used to investigate the role of modified histones, specifically acetylated and methylated histone H3, in rDNA-PE. A model is proposed in which the strength of silent chromatin within an rDNA repeat unit is determined by the amount of Sir2 associated with the repeat. The association of Sir2 correlates directly with the strength of silent chromatin in an rDNA repeat; more silent rDNA repeat units are associated with more Sir2, less silent rDNA repeat units are associated with less Sir2 (Figure 3-13).

3.4.2 Mechanisms to regulate rDNA-PE

Acetylated histones have regulatory roles in both gene expression and silent chromatin. Sir2 deacetylase activity is required for silent chromatin at the rDNA [30]; without Sir2 activity the levels of acetylated histone H3 increase at the rDNA, as do Pol II gene expression and recombination [18, 26, 28]. The strength of rDNA silencing is dependent on Sir2 dosage [36]; when Sir2 levels are increased or decreased in the cell, the strength of rDNA silent chromatin changes. The ChIP experiments presented here show that rDNA-PE in NTS2 is a result of variation in Sir2 association with specific rDNA repeats. Less Sir2 associates with the *HIS3*-marked repeats that are highly transcribed, and more Sir2 associates with *HIS3*-marked rDNA repeats that are poorly transcribed (Figure 3-9). This correlation is in agreement with the previously reported dosage-effect of cellular Sir2 on rDNA silent chromatin; more Sir2 results in stronger rDNA silencing [36]. Our data show this principle applies to individual rDNA repeats. Previous reports show that in cells lacking Sir2, the level of acetylated histones across the rDNA array increases [22, 35]; ChIP experiments assessing levels of acetylated

A.



B.

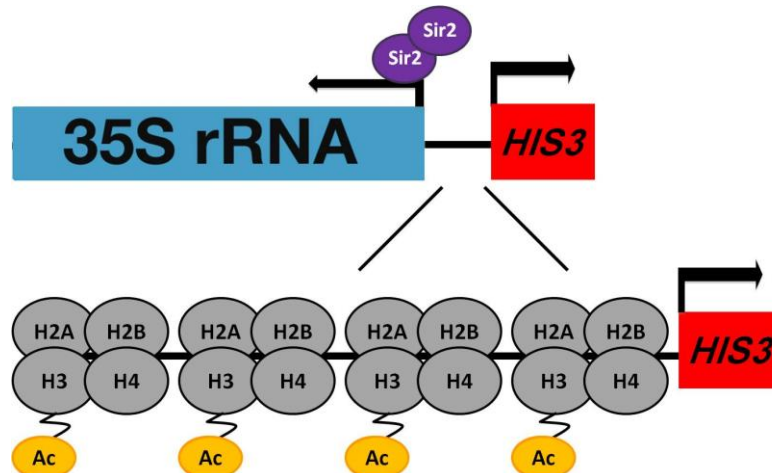


Figure 3-13. More Sir2 associates with NTS2 in more silent rDNA repeats, resulting in rDNA-PE. A partial single rDNA repeat unit is shown, containing the 35S rRNA gene and the *HIS3* gene integrated into NTS2. Arrows indicate direction of transcription. The promoter region of *HIS3* is magnified to show representative histone octamers composed of two copies each H2A, H2B, H3 and H4. Lysine residues in the tail of histone H3 are deacetylated by Sir2. Ac, acetyl group on tail of histone H3. (A) A silent rDNA repeat unit with high levels of Sir2 associating with the *HIS3*-marked rDNA repeat. Low levels of AcH3 are present, and a low level of *HIS3* transcript is produced. (B) A less silent rDNA repeat is shown with low association of Sir2 with the *HIS3*-marked repeat, leading to higher levels of AcH3 and more *HIS3* transcript.

histone H3 in *HIS3*-marked rDNA repeats correlate with differences in Sir2 association, indicating that differences in Sir2 association with individual rDNA repeats can lead to a repeat-specific change in chromatin structure.

Future experiments to determine how Sir2 association with NTS2 varies in different rDNA repeat units will focus on the role of Pol I activity. The RENT complex is recruited to NTS2 through Net1-Pol I interactions [22, 39], and a reciprocal relationship has been reported between Pol I and Pol II activity in the rDNA array. When the number of rDNA repeat units is decreased, the density of Pol I activity increases and Pol II silencing drastically increases, hypothesized to be the result of the increase in Pol I density at the rDNA array [55]. To determine if this reciprocal relationship acts in a repeat-specific manner, a *HIS3* reporter integrated in an rDNA repeat close to the Pol I binding site, but not interfering with Pol I regulatory sequences, will be analyzed by ChIP to determine the effect of Pol I transcription on Pol II silencing within a single rDNA repeat. If Pol I has a role in rDNA-PE it is expected, based on the reciprocal silencing model proposed by Cioci *et al.*, that *HIS3*-marked rDNA repeats with high Pol II transcription activity will have consistently less Pol I than other repeats. These strains may also provide insight into the frequency of transcription activity of Pol I in a particular rDNA repeat and may show if some rDNA repeat units are more highly transcribed by Pol I than other rDNA repeats.

Additionally, the effect of the autonomous replicating sequence (ARS) on rDNA-PE may be investigated. Each rDNA repeat unit contains an ARS; microscopy experiments have shown a random pattern of ARS usage in the rDNA array, suggesting

that origins are used stochastically [16]. If this is the case, ARS activity is unlikely to be a regulator of rDNA-PE. However, the firing of an ARS is repressed by Sir2; it has been reported that ARS usage in the rDNA doubles in *sir2Δ* cells compared to *SIR2⁺* cells [16]. It is possible that use of an individual ARS is regulated by the level of Sir2 associated with an rDNA repeat. However, the firing of less silent repeats may not dictate an overall non-random, specific pattern detectable by microscopy. Most of the sixteen NTS2::*HIS3* strains used in this study had strong silencing, while three strains were less silent. It is possible that the ARS in a less silent rDNA repeat is used consistently while the ARS in a more silent repeat is used randomly. If the majority of rDNA repeats are strongly silenced, an overall random pattern of ARS firing would be expected. Nonetheless, the presence of the single *HIS3*-marked repeat may facilitate studies aimed at understanding the role of Sir2 in the firing of an individual ARS.

Data from ChIP experiments with NTS1::*HIS3* strains suggest variation in the strength of silent chromatin in NTS1 is regulated by a factor(s) other than Sir2. Data from other researchers support that silent chromatin is regulated differently in NTS1 than in NTS2, so it is not unexpected that rDNA-PE would be regulated differently. The recruitment of Sir2 and Net1 to NTS1 occurs through interaction of Net1 with the protein Fob1 at the replication fork barrier region. Deletion of Fob1 abolishes silent chromatin in NTS1 but has no effect on the association of Net1 and Sir2 with NTS2 [22], making Fob1 a potential candidate for regulating rDNA-PE in NTS1. Fob1 is able to oligomerize [68], which has been shown to play a regulatory role in chromosome interactions that promote Fob1-dependent recombination within the rDNA [67]. The

effect of Fob1 oligomerization on Pol II silencing and RENT association in NTS1 has not been studied, but the oligomerization that influences intrachromatid recombination could affect the strength of Pol II transcriptional silencing in NTS1. The roles of Fob1 in arrest of replication forks and in silent chromatin are considered separate functions [142]. Studies looking at Pol II transcriptional silencing in Fob1 oligomerization mutants may provide insight into the role of Fob1 in regulating Pol II transcriptional silencing in NTS1.

3.4.3 Role of Set1 and methylated histones in rDNA-PE

Tri-methylation of lysine 4 of the tail of histone H3 (H3K4) is often associated with actively transcribed genes [136], but a role for H3K4 methylation in rDNA silent chromatin has also been established [35, 41, 135]. Sir2 and the histone methyltransferase Set1 have been shown to silence the rDNA independently [35]. While loss of Sir2 has a drastic effect on rDNA silent chromatin in the form of highly increased recombination and Pol II transcription, loss of Set1 has little effect on recombination rate and only a two- to three-fold increase in Pol II transcription. In cells lacking Sir2 activity, methylation of H3K4 increases five- to six-fold in the NTS2 region of the rDNA [18]. To explain the relationship between Sir2 and Set1 activity, it was proposed that Sir2 activity excludes H3K4 methylation from the rDNA. By deleting Set1 in selected NTS2::*HIS3* strains, the effect of H3K4 methylation on Pol II transcriptional silencing in individual rDNA repeat units was assessed in this study. Strains lacking Set1 retain variation in gene silencing at the rDNA, suggesting that Set1 and H3K4 methylation do not regulate rDNA-PE.

Unexpectedly, a *set1Δ* strain (MBY2962) had an increase in Pol II silencing compared to the *SET1*⁺ parent strain (Figure 3-7, Table 3-4); the level of *HIS3* transcript was reduced by approximately half in *set1Δ* cells compared to the *SET1*⁺ parent strain. The *set1Δ* strain was derived from MBY2608, the least silent NTS2::*HIS3* strain that had the least amount of Sir2 associated with the *HIS3*-marked rDNA repeat. This begs the question, do the levels of Sir2 and AcH3 in an rDNA repeat influence the effect of H3K4 methylation on silent chromatin within that rDNA repeat? The unexpected increase in *HIS3* silencing suggests that the effect of H3K4 methylation on Pol II transcriptional silencing may vary in different rDNA repeats in a Sir2-responsive manner.

Previous reports show that Sir2 activity, resulting in lower levels of AcH3, excludes H3K4 methylation from the *S. cerevisiae* rDNA array [18]. Others have proposed that a decrease in methylated H3K4 results in less AcH3 [143]. In actively transcribed genes, proper histone deacetylation by the complex Rpd3S in coding regions is dependent on methylation of H3K36 by Set2 [144]. Documented interactions between histone methylation and acetylation support that interplay between Sir2 deacetylase activity and Set1 methylation of H3K4 is quite possible in a silenced region like the rDNA where deacetylation of histones is important to maintain a repressive chromatin structure. Less Sir2 activity in an rDNA repeat would allow for increased AcH3, as in MBY2608, and would be expected to be permissive for higher levels of H3K4 methylation in that rDNA repeat. The loss of H3K4 methylation may allow for, or even encourage, increased activity or recruitment of Sir2 within that single rDNA repeat, resulting in an increased strength of Pol II transcriptional silencing as is observed in

MBY2962 (*set1Δ*) compared to MBY2608 (*SET1*⁺). Our data showing that Sir2 association is higher in more silent rDNA repeats support the idea that change in chromatin structure can occur in a repeat-specific manner. In other rDNA repeats with higher levels of Sir2, and therefore lower levels of AcH3 and, presumably, lower levels of methylated H3K4, the loss of H3K4 methylation may not increase Sir2 activity with that rDNA repeat; however, the silencing role of H3K4 methylation is reduced resulting in a two- to three-fold loss of Pol II transcriptional silencing. The association of Sir2 or modified histones with the *HIS3*-marked repeats in *set1Δ* strains is a potential area for future investigation.

3.4.4 Memory of rDNA silent chromatin

The RNA analysis reported herein revealed a low standard deviation for the average *HIS3/ACT1* mRNA ratio among replicate experiments, indicating that the strength of silent chromatin in a single rDNA repeat unit is stable. It has been shown by microscopy that Sir2 leaves the rDNA at the end of mitosis, so it is likely that rDNA silent chromatin is re-established each cell cycle as Sir2 returns to the rDNA [37]. Additionally, after Sir2 was depleted from the cell using a Tet-off system and then re-expressed after removal of doxycycline, the newly established silent chromatin was similar to that in cells in which Sir2 was not depleted; re-setting was observed in several strains with marked rDNA repeats that had different strengths of silent chromatin. This result also suggests that although Sir2 is necessary for silent chromatin, Sir2 does not determine the strength of silent chromatin within a single rDNA repeat unit. This means that another factor(s) associated with the rDNA determines the strength of rDNA silent

chromatin, possibly through altering chromatin structure, protein recruitment, or by regulating the activity or association of Sir2 with the rDNA in a repeat-specific manner. Identification of this factor may provide information as to how rDNA-PE is regulated. It should be recognized that the mechanism controlling memory of the strength of silent chromatin within an rDNA repeat unit could be different in NTS2 and NTS1, consistent with the hypothesis that the regulation of silent chromatin is different in NTS2 and NTS1.

Fob1 affects silent chromatin in NTS1 but not in NTS2 [22]. Levels of Fob1 have been shown to fluctuate with the cell cycle [145], an observation making Fob1 an unlikely candidate to provide stability to the strength of silent chromatin. Net1, responsible for recruiting Sir2 to the rDNA, has been shown by microscopy to associate with the rDNA throughout the cell cycle [37, 38] and could be a candidate for determining the strength of silent chromatin in NTS2. However, Net1 is recruited to NTS1 by Fob1 [22]; therefore, it is unlikely that Net1 association with NTS1 is stable throughout the cell cycle. Because Pol I interacts with Net1 in NTS2 [22, 39], and Pol I transcription is not regulated by the cell cycle [146], the microscopy data showing Net1 associated with the rDNA throughout the cell cycle would have detected Net1 at NTS2 even if Net1 was depleted in NTS1.

Various histone modifications influence the inheritance and determination of chromatin states in many organisms [6, 97, 143, 147, 148], so these modifications should not be ignored when considering possible factors for controlling memory of rDNA silent chromatin. In addition to acetylation and methylation, ubiquitination has been

implicated in chromatin function at all silent chromatin domains in *S. cerevisiae* [26, 149]. Additional studies specifically identified ubiquitinated histones; one example is that the ubiquitination of lysine 123 of H2B by Rad6 is needed for H3K4 methylation by Set1 of the COMPASS complex; lack of ubiquitinated H2B compromises telomeric silencing [98, 150]. Further, rDNA recombination rate increases twelve- to seventy-fold in *rad6Δ* cells [26]. Loss of Set1-mediated H3K4 methylation results in only a two- to three-fold increase in rDNA recombination, far less than the reported twelve- to seventy-fold increase in recombination in *rad6Δ* cells, suggesting that Rad6 ubiquitination may be orchestrating rDNA silent chromatin in more ways than just through H3K4 methylation, making this epigenetic modification a potential candidate for involvement in rDNA-PE. Studies investigating the chromatin landscape in different rDNA repeats are necessary to determine if cross-talk between modified histone residues could possibly dictate rDNA-PE through Net1 or other silencing proteins.

Several proteins have been shown to interact with the rDNA to tether the array to the inner nuclear membrane and regulate rDNA silent chromatin. Pol II silencing and mitotic recombination are both affected by the proteins required for tethering of the rDNA to the inner nuclear membrane; many of the tethering proteins have been shown to affect one or both of these silent chromatin functions [56]. The effect of spatial constraints and three-dimensional architecture of the rDNA on silent chromatin has yet to be studied in depth, but these factors could play a critical role in the stability of the rDNA locus and formation of silent chromatin structure.

3.4.5 Summary

Taken together, the data indicate that association of Sir2 with an individual rDNA repeat is the major contributing factor in determining the strength of silent chromatin within an individual rDNA repeat. However, it cannot be ignored that regulation of silent chromatin in some rDNA repeat units is multi-faceted and more complex. Because the stability of the rDNA is important to cell health, this is not unreasonable. The rDNA may be a balancing act of more and less silent repeats. Variation in silent chromatin could be a mechanism by which the cell can keep this huge array stable (in silent repeats) yet respond quickly to stress or environmental changes in which recombination or an increase in ribosomal transcript is necessary (in less silent repeats).

The strains used in this study provide an avenue to investigate the relationship between histone modifications within a single rDNA repeat unit and can provide a model system for studies about the impact of histone modifications on the presence or absence of other modifications (histone crosstalk, or the histone code) in silent chromatin. In addition, the activity of Pol I and Pol III within an rDNA repeat unit could also be changing the chromatin landscape, particularly if a repeat is always, or in contrast never, transcribed by either of these polymerases. Tethering of the rDNA to the inner nuclear membrane could also be playing a role if some repeats were consistently closer or farther from the point of tethering. Experiments to investigate these possibilities can be performed using the strain collections generated during this study to gain insight into the regulation of silent chromatin in individual rDNA repeats. The data presented here shed

light on a previously unappreciated aspect of rDNA silent chromatin and show that regulation of silent chromatin in repeated DNA sequences is a complicated puzzle, but the strains used in this study provide the tools needed to piece it together.

IV. LOSS OF SET1 METHYLTRANSFERASE ACTIVITY CONFERS SENSITIVITY TO 3-AMINO-1,2,4-TRIAZOLE IN *Saccharomyces cerevisiae*

4.1 INTRODUCTION

Modifications to histone tails play a critical role in regulating transcription in euchromatin and repressing transcription in heterochromatin. One modification in the budding yeast *Saccharomyces cerevisiae* that has been shown to both promote and repress transcription is methylation of the fourth lysine residue on the tail of histone H3 (H3K4). The lysine methyltransferase responsible for mono-, di-, and tri-methylation of H3K4 in *S. cerevisiae* is Set1 [41]. Strikingly, cells are still quite healthy after the deletion of *SET1*. Only recently have phenotypes been reported in cells lacking Set1, and the phenotypes occur under stressed growth conditions [151, 152].

Methylation of H3K4 plays a role in silent chromatin at the ribosomal DNA (rDNA) locus [35, 41, 135]. Loss of H3K4 methylation results in a three-fold increase in transcript of a silenced Ty1*his3AI* element integrated into the rDNA array [35]. A survey of Set1 single amino acid substitution mutants has identified residues within Set1 required for different methylation specificities; the effect of those H3K4 methylation states on rDNA silencing was also analyzed [135].

To simplify screening of Set1 mutants for use in an undergraduate teaching laboratory, a spot plate analysis was developed using yeast strains containing a single *HIS3* gene integrated into the rDNA array and Set1 mutants with different methylation capabilities, and therefore different strengths of rDNA silent chromatin. Growth

differences were expected to be detected on synthetic complete media lacking histidine (sc-his) containing 3-Amino-1,2,4-triazole (3-AT), an herbicide that is a competitive inhibitor of the *HIS3* gene product, imidazoleglycerol-phosphate dehydratase [153, 154]. In cells having a Set1 mutant that is unable to silence the *HIS3* gene in the rDNA array, more growth was expected than in cells retaining Set1 activity that silences *HIS3* in the rDNA array.

Growth of Set1 mutants on sc-his + 3-AT resulted in detectable growth inhibitions, however the results were surprising. Cells lacking Set1 function, therefore having reduced rDNA silencing (more *HIS3* transcript), actually grew less robustly than cells with rDNA silencing (less *HIS3* transcript). This growth pattern was observed when *HIS3* was located on chr *XII* outside of the rDNA array and at the endogenous location of *HIS3* on chr *XV*, indicating sensitivity to 3-AT is not due to *HIS3* transcription dictated by rDNA silent chromatin.

Changes in nucleosome positioning across the genome have been documented in cells in the presence of 3-AT [155-158]. These changes have not been assessed in the absence of Set1 activity. It has recently been shown that H3K4 methylation can dictate the activity of the chromatin remodeler Swr1 at stress-response genes [152]. Data presented herein, in combination with published data, are consistent with the idea that H3K4 methylation plays a role in chromatin remodeling in the presence of 3-AT, and that without H3K4 methylation cells are not capable of making the chromatin structure changes necessary to overcome the stress of 3-AT, resulting in a sensitivity of cells lacking Set1 function.

4.2 MATERIALS AND METHODS

4.2.1 Media

Growth media used in these experiments has been described previously [123]. YPADTU is YPD media supplemented with 40 mg/L adenine hemisulfate, 80 mg/L L-tryptophan, and 20 mg/L uracil. When indicated, 1 M 3-Amino-1,2,4-triazole (3-AT) in water was added to media at a concentration of 10 mM prior to pouring plates used for spot growth analysis.

4.2.2 Yeast strains and plasmids used in this study

All yeast strains constructed and used in this study are listed in Table 4-1. Plasmids containing *set1* mutant alleles used in this study have been described previously [135]. Construction of strains and verification of the integration of a single copy of pRS406, pRS406-*set1* mutants, and pRS406-*SET1*⁺ plasmids in each strain was performed as previously described [135].

Plasmids containing *HHT2-HHF2* and *hht2-K4R-HHF2* have been described previously [41], except that the plasmids used in this study were marked with *TRP1* for selection of transformants. Plasmid was transformed into cells from 1 ml saturated culture as previously described except that 40 µg single-stranded salmon sperm carrier DNA was used [159]. Cells were plated to sc-trp agar media and incubated at 30°C until single colonies appeared.

4.2.3 Plate growth analysis

Inhibition of growth by 3-AT is dose-dependent; the optimal concentration (10 mM) was determined by evaluating growth of yeast cells on sc-his agar plates with three

Table 4-1. Yeast strains used in this study.

Strain	Genotype	<i>set1</i> activity ^a
MBY 2942	<i>MAT_a</i> (<i>his3</i> Δ200) <i>leu2</i> Δ(0 or 1) <i>lys2-128</i> δ <i>trp1</i> Δ63 <i>ura3-52 set1</i> Δ::KANMX4 NTS2::HIS3	
MBY 2945	MBY 2942 <i>ura3-52::pRS406-set1-Y967A</i>	none
MBY 2946	MBY 2942 <i>ura3-52::pRS406-set1-Y967F</i>	mono
MBY 2965	MBY 2942 <i>ura3-52::pRS406-SET1</i> ⁺	WT
MBY 2967	MBY 2942 <i>ura3-52::pRS406</i>	Δ
MBY 2968	MBY 2942 <i>ura3-52::pRS406-set1-R1013H</i>	mono/di
MBY 2970	MBY 2942 <i>ura3-52::pRS406-set1-H1017A</i>	hyper
MBY 2972	<i>MAT_a</i> <i>his3</i> Δ200 <i>leu2</i> Δ(0 or 1) <i>met15</i> Δ0 <i>trp1</i> Δ63 <i>ura3-52 set1</i> Δ::KANMX4 <i>chr XII::HIS3</i>	
MBY 2980	MBY 2972 <i>ura3-52::pRS406</i>	Δ
MBY 2981	MBY 2972 <i>ura3-52::pRS406</i>	Δ
MBY 2982	MBY 2972 <i>ura3-52::pRS406-SET1</i> ⁺	WT
MBY 2984	MBY 2972 <i>ura3-52::pRS406-set1-Y967F</i>	mono
MBY 2986	MBY 2972 <i>ura3-52::pRS406-set1-R1013H</i>	mono/di
MBY 2989	MBY 2972 <i>ura3-52::pRS406-set1-H1017A</i>	hyper
MBY 2990	MBY 2972 <i>ura3-52::pRS406-set1-Y967A</i>	none
MBY 1590	<i>MAT_a</i> <i>lys2-128</i> δ <i>trp1</i> Δ63 <i>ura3-52 set1</i> Δ::KANMX4	
MBY 2992	MBY 1590 <i>ura3-52::pRS406</i>	Δ
MBY 2994	MBY 1590 <i>ura3-52::pRS406-SET1</i> ⁺	WT
MBY 2996	MBY 1590 <i>ura3-52::pRS406-set1-R1013H</i>	mono/di
MBY 2998	MBY 1590 <i>ura3-52::pRS406-set1-Y967A</i>	none
MBY 3000	MBY 1590 <i>ura3-52::pRS406-set1-Y967F</i>	mono
MBY 3002	MBY 1590 <i>ura3-52::pRS406-set1-H1017A</i>	hyper
MBY 3029	<i>Mata</i> <i>lys2-128</i> δ <i>trp1</i> Δ63 <i>ura3-52 set1</i> Δ::KANMX4 (<i>hht1-hhf1</i>)Δ::LEU2 (<i>hht2-hhf2</i>)Δ::KANMX4 <i>pHHT2-HHF2-TRP1-CEN</i>	
MBY 3030	<i>Mata</i> <i>lys2-128</i> δ <i>trp1</i> Δ63 <i>ura3-52 set1</i> Δ::KANMX4 (<i>hht1-hhf1</i>)Δ::LEU2 (<i>hht2-hhf2</i>)Δ::KANMX4 <i>phht2-K4R-HHF2-TRP1-CEN</i>	
MBY 3031	MBY 3029 <i>ura3-52::pRS406-SET1</i> ⁺	WT
MBY 3032	MBY 3029 <i>ura3-52::pRS406-set1</i> Δ	Δ
MBY 3033	MBY 3029 <i>ura3-52::pRS406-set1-Y967A</i>	none

Table 4-1 Continued.

Strain	Genotype	<i>set1</i> activity^a
MBY 3034	MBY 3029 <i>ura3-52::pRS406-set1-Y967F</i>	mono
MBY 3035	MBY 3029 <i>ura3-52::pRS406-set1-R1013H</i>	mono/di
MBY 3036	MBY 3029 <i>ura3-52::pRS406-set1-H1017A</i>	hyper
MBY 3037	MBY 3030 <i>ura3-52::pRS406-SET1⁺</i>	WT
MBY 3038	MBY 3030 <i>ura3-52::pRS406-set1Δ</i>	Δ
MBY 3039	MBY 3030 <i>ura3-52::pRS406-set1-Y967A</i>	none
MBY 3040	MBY 3030 <i>ura3-52::pRS406-set1-Y967F</i>	mono
MBY 3041	MBY 3030 <i>ura3-52::pRS406-set1-R1013H</i>	mono/di
MBY 3042	MBY 3030 <i>ura3-52::pRS406-set1-H1017A</i>	hyper

^a*set1* mutants made and *set1* activity determined in Williamson *et al.* 2013.

concentrations of 3-AT (5 mM, 10 mM, or 20 mM). For plate growth analysis, cultures of yeast cells were grown to saturation at 30°C in YPADTU liquid medium. Eight, five-fold serial dilutions were made in sterile water. The last six dilutions were plated (5 µl) on each of four types of agar plates: sc-his, sc-his + 10 mM 3-AT, sc-complete, sc-complete + 10 mM 3-AT. For preliminary experiments with yeast strains harboring H3K4 or H3K4R on a plasmid, six, ten-fold dilutions were made in sterile water and plated (5 µl) to each of the four media types listed above. Plates were incubated at 30°C. Five-fold dilutions were imaged after 24 and 42 or 44 hours; ten-fold dilutions were imaged after 33 hours.

4.2.4 Northern blot analysis

Saturated cultures of yeast strains grown in sc-his or sc-his + 10 mM 3-AT liquid medium were diluted into 10 ml fresh sc-his or sc-his + 10 mM 3-AT at $\sim 4 \times 10^6$ cells/ml and grown at 30°C to a density of $1-2 \times 10^7$ cells/ml. Total RNA was extracted from yeast cultures as previously described [129] with the following modifications. Cells were resuspended in 450 µl AE buffer (50 mM $C_2H_3NaO_2$ pH 5.3, 10 mM EDTA) transferred to a microfuge tube, and 50 µl 10% SDS was added. An extraction with an equal volume of chloroform:isoamyl alcohol (24:1) was done before the addition of 50 µl 3M sodium acetate pH 5.3 and 2.5 volumes 100% ethanol. After precipitation RNA was resuspended in 20-30 µl sterile water and stored at -70°C. RNA (20 µg) was analyzed by Northern blot as previously described [130]. ^{32}P -labelled DNA probes synthesized by random priming were hybridized to membranes to measure the steady state level of *HIS3* transcript. A ^{32}P -labelled DNA probe specific for *ACT1* was used as a loading control.

Northern blots were imaged with a Pharos FX Plus Molecular Imager and quantified with Quantity One software (Bio-Rad).

4.3 RESULTS

4.3.1 Sensitivity of cells lacking Set1 activity grown on sc-his + 3-AT

Loss of Set1 activity and some changes in Set1 product specificity result in reduced silencing of the Pol II-transcribed Ty1*his3AI* gene integrated into the rDNA array [35, 41, 135]. The effect of *set1* mutants on expression of the Ty1*his3AI* gene cannot be evaluated simply and quantitatively by growth on agar plates. Therefore, yeast strains with a *HIS3* gene integrated into the rDNA locus were constructed and used in plate growth assays to test the effect of different *set1* mutants on *HIS3* gene expression. Plasmids containing different *set1* mutant genes were integrated into the *ura3-52* locus of a *set1*Δ yeast strain (MBY2942) that has a single *HIS3* gene integrated into the NTS2 portion of one rDNA repeat unit. When expressed in yeast, each of the Set1 mutants has been previously shown to have different H3K4 methylation capabilities: Y967A, no methylation; Y967F, mono-methylation only; R1013H, mono- and di-methylation only; H1017A, hyper-methylation for all three product specificities [135]. In addition, derivatives of yeast strain MBY2942 were made that contain a *SET1*⁺ (WT mono-, di- and tri- methylation) plasmid and *set1*Δ (empty vector, no methylation) at the *ura3-52* locus. The effects of changes in Set1 function were then tested in the yeast strains using plate growth assays on solid agar medium.

Saturated cultures were serially diluted and spotted to four solid agar media types as described in Materials and Methods. After 24 and 44 hours of growth, all strains grew similarly on sc-his media, indicating that alterations in *HIS3* gene expression due to changes in Set1 methylation activity could not be detected by growth on sc-his media (Figure 4-1, top panels). This was not unexpected because previous work has revealed that *HIS3* mRNA levels reduced to 10% to 15% of the wild-type level permits a wild-type level of growth on sc-his media (R.A.J. unpublished data). No growth differences were seen on sc-complete media, indicating that an equal number of cells were plated in each dilution of each yeast strain and that no strains have a growth deficiency in rich media.

The herbicide 3-AT is a competitive inhibitor of the *HIS3* gene product, imidazoleglycerol-phosphate dehydratase, and can be used to exacerbate growth deficiencies due to reduced *HIS3* expression. In contrast to the sc-his spot plates, there is very little growth after 24 hours on sc-his + 3-AT, indicating that 3-AT does reduce growth, presumably by reducing the amount of imidazoleglycerol-phosphate dehydratase available for histidine biosynthesis. After 44 hours, differences in growth among strains could be seen and a surprising result was observed. The strains lacking Set1 activity that have a *HIS3* gene in the rDNA (*set1Δ* and *set1*-Y967A) grew less well on 3-AT than the yeast strains that retain Set1 activity (*SET1*⁺, *set1*-Y967F, *set1*-R1013H, *set1*-H1017A) (Figure 4-1, third panel). This result was surprising, as previous Northern data showed that *set1Δ* strains with *HIS3* in the rDNA have higher *HIS3* transcript levels than *SET1*⁺

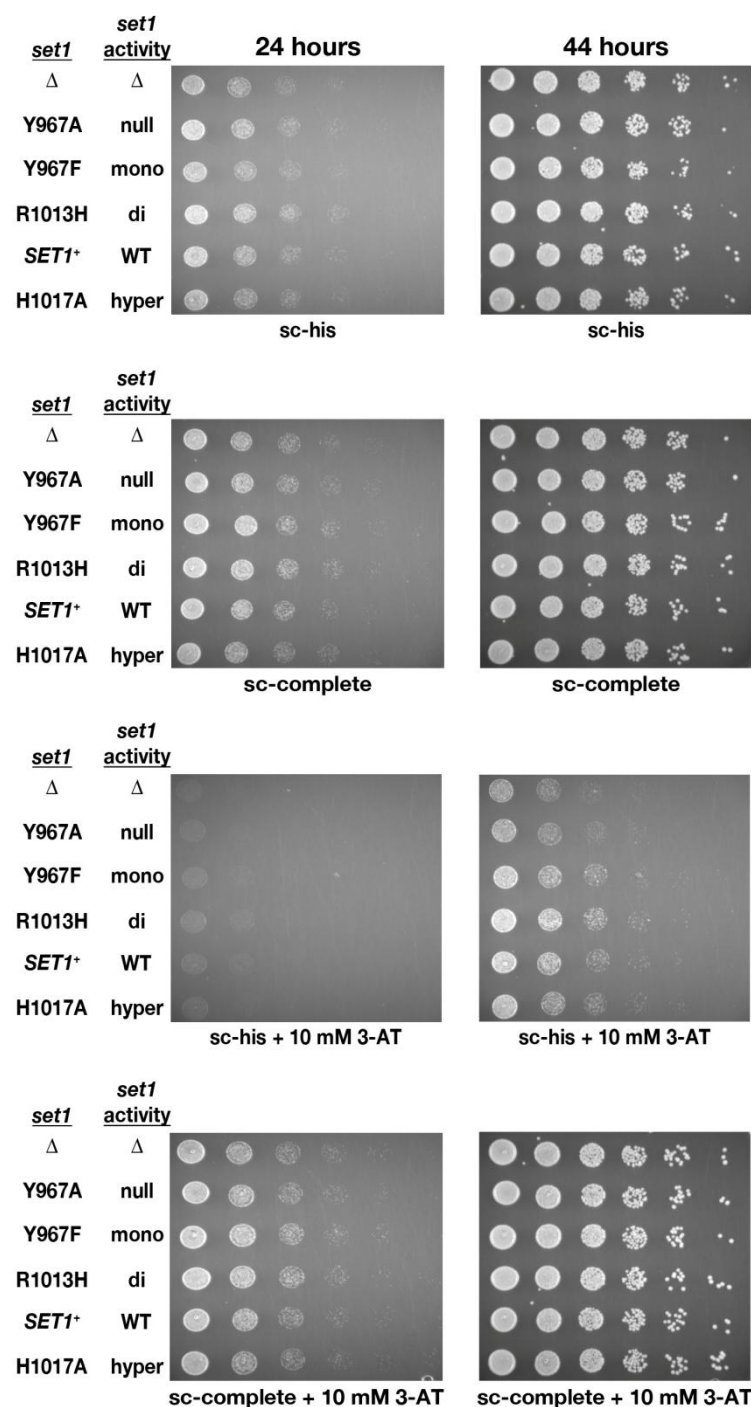


Figure 4-1. Growth defect of rDNA::HIS3 cells lacking functional Set1 on sc-his + 3-AT. Five-fold serial dilutions were spotted to sc-his, sc-his + 10 mM 3-AT, sc-complete, and sc-complete + 10 mM 3-AT, grown at 30°C, and imaged after 24 and 44 hours. The *SET1* allele present in the yeast strains and H3K4 methylation activity are indicated on left. Media type is specified below each panel.

cells (Figure 3-7, Table 3-4); it should be noted that cells used in Northern blot experiments in Figure 3-7 were grown in rich media. Serial dilutions of yeast cultures spotted on sc-complete + 3-AT (Figure 4-1, bottom panel) grew well after 24 hours, indicating that 3-AT does not confer a growth defect when histidine is present in the agar.

To test if the surprising lack of growth of the *set1Δ* and Y967A strains on sc-his + 3-AT media is due to the location of *HIS3* in the rDNA, a second collection of strains containing the Set1 mutants was made with *HIS3* located in a euchromatic region of chr *XII* outside of the rDNA array. When grown in rich media, transcription of *HIS3* outside of the rDNA array is not affected by Set1 activity; this has been shown in previous Northern blots comparing *HIS3* transcript level in a *set1Δ* strain to a *SET1*⁺ strain with *HIS3* in the same euchromatic location on chr *XII* (Figure 3-7).

Dilutions of saturated cultures were spotted to four types of solid agar, and imaged after 24 and 44 hours growth at 30°C. No growth differences were observed among the strains plated on the sc-his, sc-complete, and sc-complete + 3-AT plates, indicating a similar number of cells were plated from each strain in each dilution and that 3-AT does not confer a growth defect in the presence of sufficient histidine (Figure 4-2). After 24 hours, there was little growth of strains on sc-his + 3-AT (Figure 4-2, third panel). After 44 hours, it was apparent that the *set1Δ* and Y967A strains, both lacking Set1 activity, grew less than the other yeast strains that retained H3K4 methylation. In addition, a slight, but consistent, difference in growth was observed when comparing the strains containing wild type *SET1*⁺ (WT) and hyper-methylation

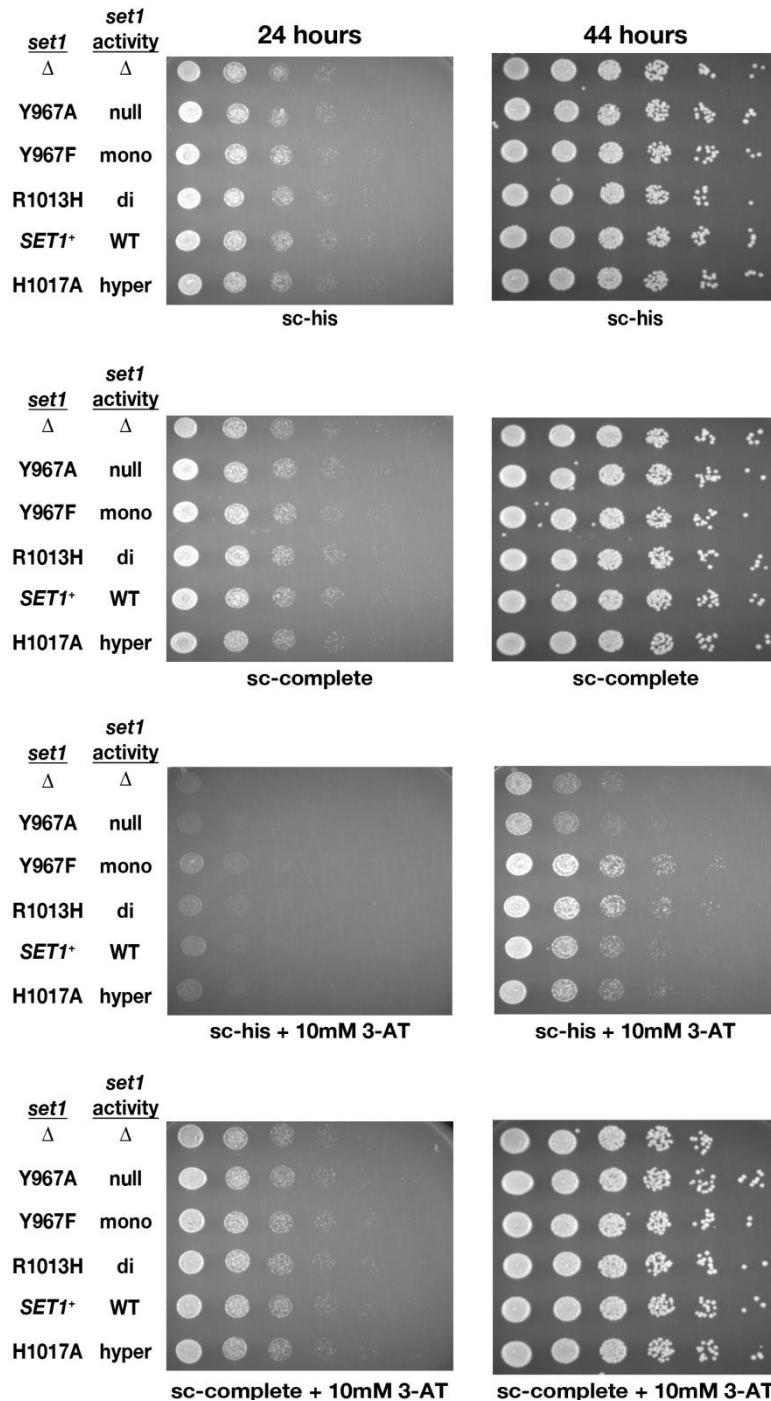


Figure 4-2. Growth defects of chr *XII::HIS3* cells lacking functional Set1 on sc-his + 3-AT. Five-fold serial dilutions were spotted to sc-his, sc-his + 10 mM 3-AT, sc-complete, and sc-complete + 10 mM 3-AT, grown at 30°C, and imaged after 24 and 44 hours. The *SET1* allele present in the yeast strains and H3K4 methylation activity are indicated on left. Media type is specified below each panel.

set1 mutant (H1017A) alleles to those with the mono-methylation (Y967F) and di-methylation *set1* mutant alleles (R1013H). Strains with H3K4 methylation capacity limited to mono- or di- methylation grew slightly better on sc-his + 3-AT agar than strains with the wild type *SET1* gene or the hyper-methylation *set1* mutant (H1017A).

To test if the genomic location of the *HIS3* gene affects the growth of cells in the presence of 3-AT, the *Set1* mutants were integrated into the *ura3-52* locus of a strain with the *HIS3* gene at its endogenous location on chr XV (MBY 1590). Dilutions of saturated cultures were plated on sc-his and sc-complete agar plates, with and without 3-AT. As was observed in yeast strains with the *HIS3* gene integrated into a euchromatic location on chr XII, all strains grew similarly on sc-his, sc-complete, and sc-complete + 3-AT (Figure 4-3). After 24 hours, there was little growth on sc-his + 3-AT agar plates. After 42 hours, the growth pattern that emerged was similar to that observed with the other strains that had *HIS3* in the rDNA and in the euchromatic region of chr XII. Cells lacking functional *Set1* (*set1Δ* and *set1*-Y967A) grow less than cells with partial or full *Set1* function. Consistent minor growth differences were seen among strains with differing *Set1* product specificity. Yeast strains expressing the Y967F (retains mono-methylation) and R1013H (retains mono- and di-methylation) *set1* mutant alleles grew slightly better than those with the H1017A (hyper methylation) and the wild type *SET1* alleles.

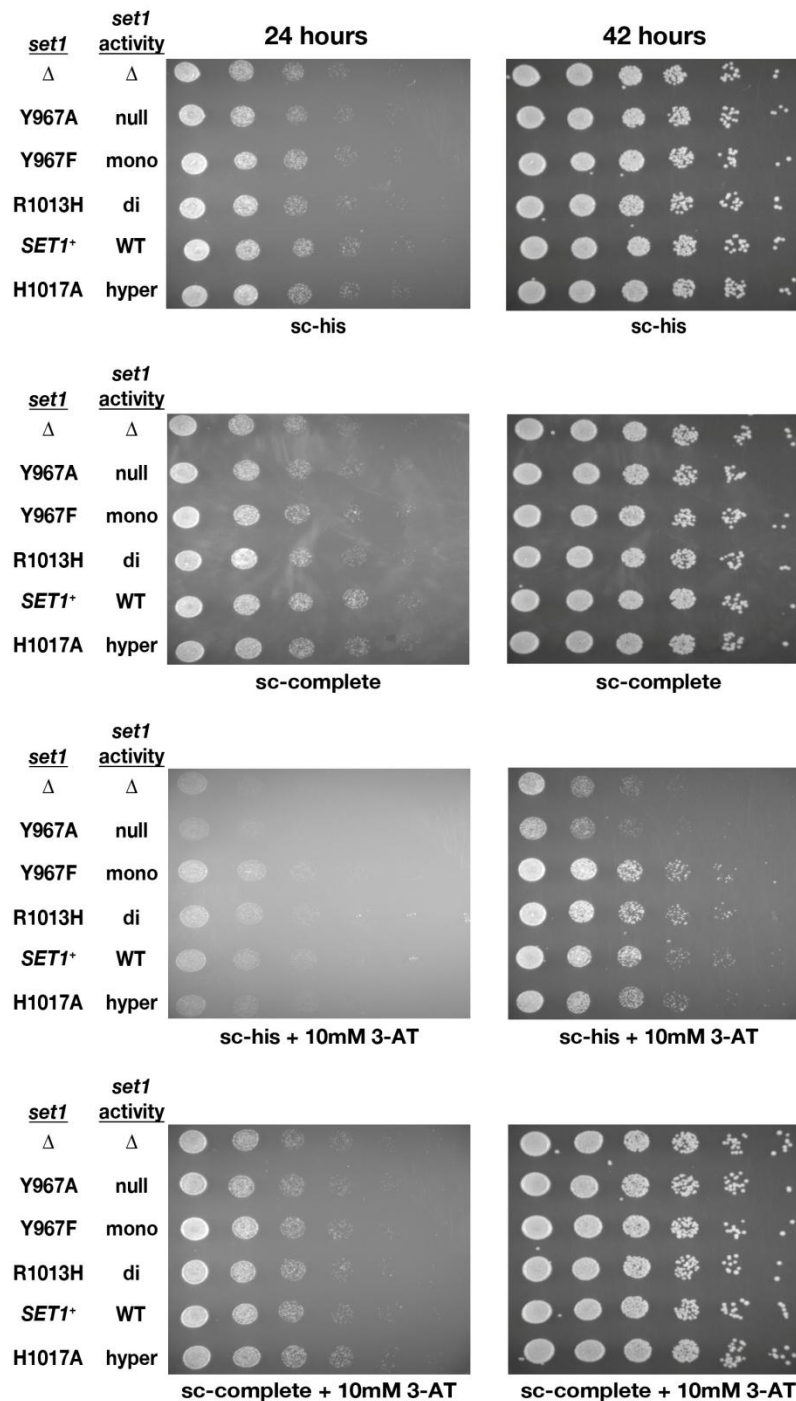


Figure 4-3. Cells lacking functional Set1 are sensitive to 3-AT on sc-his medium. Five-fold serial dilutions were spotted to sc-his, sc-his + 10 mM 3-AT, sc-complete, and sc-complete + 10 mM 3-AT, grown at 30°C, and imaged after 24 and 42 hours. The *SET1* allele present in the yeast strains and H3K4 methylation activity are indicated on left. Media type is specified below each panel.

4.3.2 *HIS3* transcript levels are reduced in cells without Set1 activity compared to cells with Set1 activity when grown in sc-his + 3-AT

After observing unexpected differences in growth on spot plates, Northern blots were performed to measure *HIS3* transcript levels in cells grown in sc-his + 3-AT medium using strains with *HIS3* in a euchromatic region of chr *XII* (Figure 4-4A). *ACT1* transcript was used as a loading control. The *HIS3/ACT1* mRNA ratio of each strain was normalized to the *HIS3/ACT1* mRNA ratio of the wild type *SET1*⁺ strain. Cultures grown in sc-his + 3-AT medium have variation in *HIS3* transcript levels that correlates with their growth on sc-his + 3AT spot plates. Strains lacking H3K4 methylation activity, *set1Δ* and Y967A, have approximately 60% of *HIS3* transcript as wild type *SET1*⁺ cells (Figure 4-4A; *HIS3/ACT1* mRNA ratio 0.58 and 0.60, respectively), indicating that Set1 function is necessary for an increased level of *HIS3* transcript when 3-AT is present. Strains retaining H3K4 methylation, Y967F, R1013H, and H1017A, have *HIS3/ACT1* mRNA ratios that are similar to those of cells with the wild type *SET1* gene (Figure 4-4A).

Cells grown in sc-his medium were analyzed to measure *HIS3* transcript levels in the absence of 3-AT. The ratio of *HIS3/ACT1* mRNA in each strain was normalized to the *HIS3/ACT1* mRNA ratio in the wild type *SET1*⁺ strain grown in sc-his medium. *HIS3* transcript levels were found to be similar in the strains when grown in sc-his medium (*HIS3/ACT1* mRNA ratio 1.0 to 1.27), indicating that *HIS3* transcription is not affected significantly by the absence of or change in Set1 function under histidine starvation conditions without the additional stress of 3-AT (Figure 4-4B).

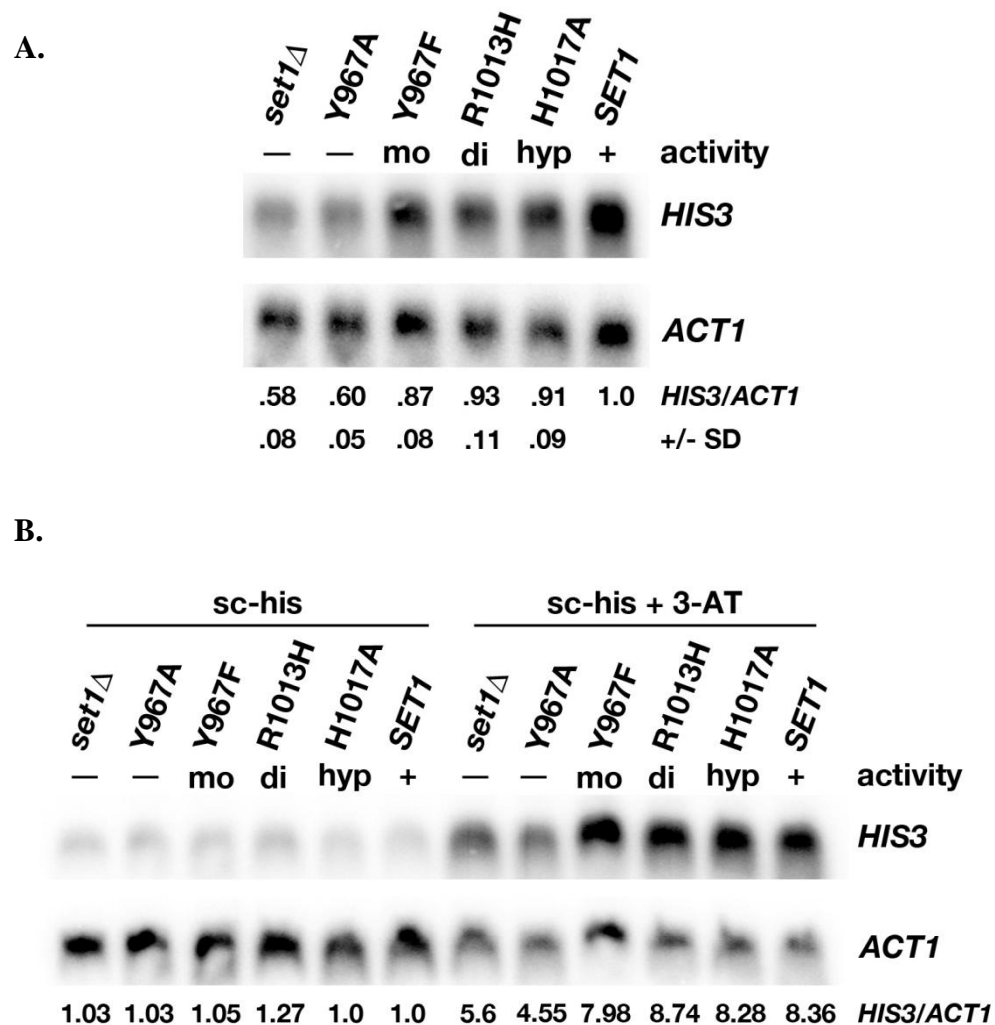


Figure 4-4. *HIS3* transcript is lower in cells lacking Set1 function. (A) Total RNA from chrXII::*HIS3* yeast strains containing *set1* Δ , *SET1*⁺, or *set1* mutants grown in sc-his + 10 mM 3-AT medium was analyzed by Northern blot. *ACT1* mRNA levels were used to normalize loading. The *HIS3/ACT1* mRNA ratios were normalized to the *HIS3/ACT1* mRNA ratio from the wild-type *SET1*⁺ yeast strain. *HIS3/ACT1* mRNA ratios of *set1* Δ and Y967A are significantly less than the *SET1*⁺ yeast strain, $p < 0.05$. Standard deviations (SD) were determined from three independent experiments. (B) Total RNA from cells grown in sc-his and sc-his + 10 mM 3-AT medium was analyzed by Northern blot. The *HIS3/ACT1* mRNA ratios were normalized to the *HIS3/ACT1* mRNA ratio from the wild-type *SET1*⁺ yeast strain grown in sc-his.

To compare *HIS3* expression in cells grown in sc-his medium to cells grown in sc-his + 3-AT medium, the *HIS3/ACT1* mRNA ratio in cells grown in sc-his + 3-AT was normalized to the wild-type *SET1*⁺ strain grown in sc-his. In cells lacking Set1 activity, an increase in *HIS3* transcript of 4.55-fold (Y967A) and 5.60-fold (*set1*Δ) was observed (Figure 4-4B). In the four strains retaining Set1 activity, the increase in *HIS3* transcript was 7.98- to 8.74-fold higher than the level in cells with wild type *SET1* grown in sc-his medium (Figure 4-4B). The 7.98 to 8.74-fold increase in *HIS3* transcript observed in cells retaining Set1 function is consistent with previously published results comparing *HIS3* transcript levels in total RNA from wild type S288C cells grown in minimal, minimal + histidine, or minimal + 3-AT media; this study reported a ten-fold increase in *HIS3* transcript in cells grown in minimal medium containing 10 mM 3-AT compared to cells grown in minimal medium without 3-AT [160]. The data in Figure 4-4 suggest that although the level of *HIS3* transcript is increased in the presence of 3-AT in strains that lack Set1 function, cells that retain the capacity to methylate H3K4 have even higher levels of *HIS3* transcript when grown in the presence of 3-AT.

4.3.3 Preliminary results indicate cells with H3K4R are sensitive to 3-AT

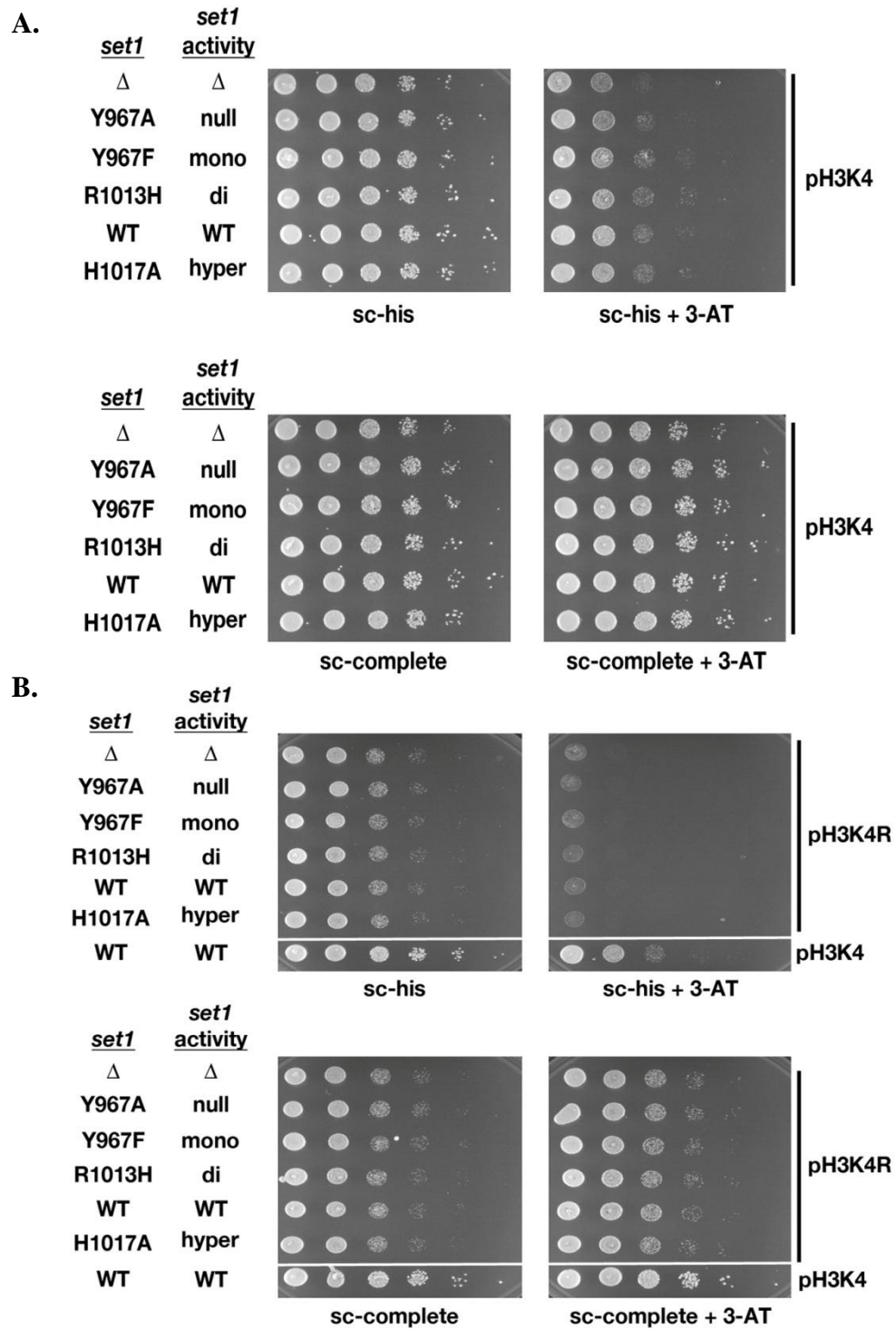
To identify if H3K4 methylation is causing sensitivity to sc-his + 3-AT medium, a collection of strains was made expressing either wild type or a mutant allele of the histone H3 encoding gene *HHT2*. Endogenous *HHT1* and *HHT2* were deleted and *HHT2* or *hht2-H3K4R* was expressed from a plasmid. The commonly used H3K4R mutant is unable to be methylated by Set1 on the fourth residue of the histone H3 N-terminal tail because the lysine has been replaced with arginine.

Six, ten-fold serial dilutions of saturated overnight cultures were plated to all four media types; plates were incubated and imaged after 33 hours growth. In cells expressing wild type H3K4, a similar growth pattern as that seen in other strain collections was observed. Strains lacking Set1 activity (*set1* Δ and *set1*-Y967A) grew less than the other strains (Figure 4-5A), although this phenotype was not as strong as was seen in previous experiments that used five-fold dilutions. On sc-his + 3-AT the growth advantage of the mono and di-methylation *set1* mutants (*set1*-Y967F and *set1*-R1013H) previously detected is not observed, however this must be confirmed in replicate experiments.

If H3K4 methylation is causing sensitivity to 3-AT, it is expected that strains expressing H3K4R will all grow as *set1* Δ cells, abolishing growth differences observed between different Set1 mutants. For comparison, a *SET1*⁺ strain expressing wild type H3K4 from a plasmid was included on each plate (Figure 4-5B, bottom row). On sc-his, sc-complete, and sc-complete + 3-AT, strains expressing H3K4R all grew similarly. Growth of H3K4R strains is reduced compared to the *SET1*⁺ H3K4 strain (Figure 4-5B, compare first six rows to the last row). On sc-his + 3-AT, all H3K4R strains are highly sensitive to 3-AT, and growth differences between Set1 mutants were abolished; this growth was consistent after 45 hours (data not shown). This growth pattern indicates that loss of H3K4 methylation confers sensitivity to 3-AT when media is lacking histidine. These preliminary growth tests will be confirmed in future experiments.

The growth defect of cells lacking Set1 activity (*set1* Δ and *set1*-Y967A) was not as drastic in the ten-fold dilutions in Figure 4-5A as was seen in previous experiments

Figure 4-5. Preliminary data indicates H3K4 methylation is important for growth on sc-his + 3-AT medium. Serial dilutions were spotted to sc-his, sc-his + 10 mM 3-AT, sc-complete, and sc-complete + 10 mM 3-AT, grown at 30°C, and imaged after 33 hours. The *SET1* allele present in the yeast strains and H3K4 methylation activity are indicated on left. Media type is specified below each panel. (A) Ten-fold serial dilutions of saturated cultures. In each strain endogenous *HHT1* and *HHT2*, which encode histone H3, are deleted and wild type histone H3 (H3K4) is expressed from a plasmid (pH3K4). (B) Ten-fold serial dilutions of saturated cultures. Endogenous *HHT1* and *HHT2* are deleted and the histone H3 mutant H3K4R is expressed from a plasmid (pH3K4R). For comparison of growth, the bottom row of each plate is a *SET1*⁺ (WT) yeast strain with pH3K4. (C) Five-fold serial dilutions of saturated cultures. Endogenous *HHT1* and *HHT2* are deleted and wild type histone H3 (H3K4) is expressed from a plasmid (pH3K4).



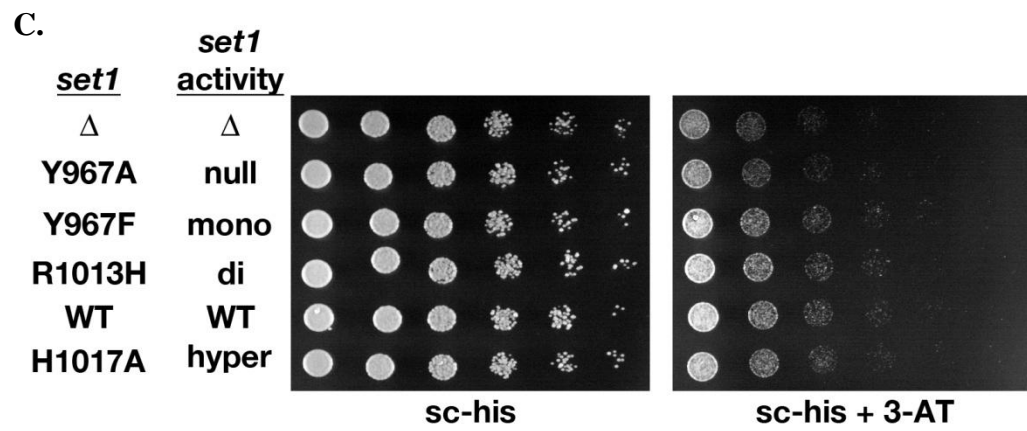


Figure 4-5. Continued.

that analyzed five-fold dilutions. To further confirm the sensitivity of *set1Δ* and *set1*-Y967A to 3-AT in these strains, five-fold dilutions of saturated cultures were made and plated to sc-his and sc-his + 3-AT media. After 33 hours growth, *set1Δ* and *set1*-Y967A grew less than the strains retaining Set1 activity, confirming the phenotype was visible when H3K4 was expressed on a plasmid (Figure 4-5C). The growth advantage of mono and di-methylation *set1* mutants (*set1*-Y967F and *set1*-R1013H) was not clearly observed. Replicate experiments will be performed to verify these observations.

4.4 DISCUSSION

The histone methyltransferase Set1 is the sole H3K4 methyltransferase in *S. cerevisiae*, and it is not essential for growth under conditions that have been tested thus far [41]. Results presented here show a phenotype resulting from sensitivity of Set1 mutants to the herbicide 3-AT in media lacking histidine. When cells are cultured on sc-his + 3-AT medium, growth is reduced and transcription of *HIS3* is approximately 40% lower in cells lacking Set1 function than in cells retaining Set1 methylation capabilities. These findings suggest that the 3-AT sensitivity may be linked to suboptimal *HIS3* transcription induction in the absence of Set1.

4.4.1 Loss of H3K4 methylation confers sensitivity to 3-AT and reduces *HIS3* transcription

The most likely candidate for conferring sensitivity to 3-AT is H3K4 methylation. Besides the well-documented role of H3K4 methylation in transcriptional regulation across species, Set1 has one known non-histone target, Dam1, which is

involved in proper chromosome segregation [161]. To test if 3-AT sensitivity is conferred through Set1 methylation of H3K4, H3K4 was replaced with H3K4R, a histone H3 mutant that cannot be methylated by Set1 at the fourth residue because the lysine is replaced with arginine. Preliminary plate growth assay results using H3K4R yeast strains support the hypothesis that loss of H3K4 methylation results in 3-AT sensitivity (Figure 4-5).

Although the specific role for H3K4 methylation in 3-AT sensitivity is still under investigation, our data suggest that sensitivity is related to the lower level of *HIS3* transcript in *set1* Δ cells in the presence of 3-AT. The Northern data in Figure 4-4 suggest that Set1 is required for maximal increase of *HIS3* transcript levels in the presence of 3-AT. Data in Figure 4-4 show that cells lacking H3K4 methylation have lower levels of *HIS3* transcript than cells with H3K4 methylation, consistent with the reduced growth of cells lacking Set1 activity on sc-his + 3-AT medium.

In support of these observations, two recent studies have shown a direct connection between H3K4 methylation and changes in gene expression that result in a phenotype under stressed growth conditions. Methylation of H3K4 by Set1 was shown to impact the ability of cells to respond to osmotic stress by controlling chromatin-remodeling complexes at stress-response genes [152]. Additionally, the hypersensitivity of *set1* Δ cells to the antifungal compound Brefeldin A (BFA) was shown to be due to altered expression of two genes caused by changes in H3K4 methylation [151].

4.4.2 Changes in chromatin structure in the presence of 3-AT

How H3K4 methylation specifically allows for maximal *HIS3* induction is a topic for future investigation, but current literature provides some direction. *HIS3* transcription is highly regulated in order to control both constitutive and inducible levels of *HIS3* mRNA [162-171]. There are major changes in nucleosome positioning across the *HIS3* gene upon induction; Gcn4 and the SWI/SNF remodeling complex are required for changes in nucleosome occupancy and positioning over the entire *HIS3* gene under induced (medium lacking histidine) growth conditions compared to uninduced (rich medium) growth conditions [156, 157]. Additional experiments confirmed the movement of nucleosomes on chromosomal *HIS3* in the presence of 3-AT [155, 158].

H3K4 methylation could be playing a key role in the nucleosome remodeling necessary for growth in 3-AT. Gcn4 has been shown to regulate hundreds of genes in many different pathways, so effects are not likely restricted to *HIS3* regulation [121]. Sensitivity to 3-AT could be a result of altered SWI/SNF association affecting expression of some genes that are induced by Gcn4. This has been shown for *ARG1*, a gene induced by Gcn4 upon amino acid starvation. The addition of 3-AT to sc-his growth medium results in increased SWI/SNF association at *ARG1* compared to cells grown on sc-his medium without 3-AT [157]. This result suggests that 3-AT causes changes in SWI/SNF association at some genes that are regulated by Gcn4. This regulation explains why there is no growth defect on sc-his media, but there is sensitivity when 3-AT is added; increased SWI/SNF recruitment that is dependent on Gcn4 occurs in the presence of 3-AT and not in sc-his media without 3-AT.

A direct link between Gcn4 and H3K4 methylation has not yet been established, but it is known that the histone acetyltransferase Gcn5, a subunit of the SAGA complex, is required for maximal induction of genes, including *HIS3*, by Gcn4 [172, 173]. H3K4 methylation could affect Gcn4 induction indirectly through Gcn5. It has been reported that histone H3 methylation affects association of the lysine acetyltransferase complex NuA4. Esa1, the lysine acetyltransferase subunit of NuA4, preferentially binds mono- and di-methylated H3K4 [174, 175]. In vivo, loss of H3K4 methylation leads to a 50% reduction in NuA4 binding to some genes, indicating that H3K4 methylation affects NuA4 recruitment in a gene-specific manner [174, 176]. NuA4 has also been shown to increase recruitment of the SAGA and SWI/SNF complexes [174, 176].

Taken together, it is hypothesized that H3K4 mono- and di-methylation can indirectly influence transcription of specific genes through the association of NuA4. Methylated H3K4 recruits NuA4, resulting in enhanced recruitment of the SAGA complex, which is associated with stronger SWI/SNF activity, leading to changes in transcript levels. In genes where Gcn4 recruits SWI/SNF in the presence of 3-AT, the loss of H3K4 methylation may reduce 3-AT-induced transcription of these genes, resulting in a sensitivity of *set1Δ* cells to 3-AT. These interactions may result in the growth advantage of mono- and di-methylation Set1 mutants in the presence of 3-AT.

This hypothesis does not have to be exclusive for genes activated by Gcn4. Nucleosomes are repositioned in many genes in the presence of 3-AT, not all of which are controlled by Gcn4; these changes can both promote and repress transcription [155]. The loss of H3K4 could be affecting remodeling at any of these genes, meaning that the

sensitivity to 3-AT may be due not only to changes in *HIS3* induction but also to changes in the induction, or repression, of a number of other genes. In addition, because sensitivity is only seen in cells required to make histidine in the presence of 3-AT, growth may be impaired due to an additive effect; that is, cells must be stressed by both histidine biosynthesis and changes in other genes caused by 3-AT in order for a phenotype to arise.

4.4.3 Summary: the role of Set1 in 3-AT sensitivity

The requirement of Set1 for growth on sc-his + 3-AT medium is a unique and unexpected role for Set1. This growth phenotype is one of a few that have been linked to Set1. Sensitivity to 3-AT may be the result of the cell's inability to induce transcription in response to 3-AT. A better understanding of the role of H3K4 methylation in response to stress, such as amino acid starvation, is expected to broaden the understanding of the impact of this important epigenetic modification in cell growth and gene expression.

V. CONCLUSIONS

5.1 SILENT CHROMATIN AT THE RIBOSOMAL DNA

The ribosomal DNA of *Saccharomyces cerevisiae* is a ~1.8 Mb, highly repeated locus that acquires a special heterochromatic structure called silent chromatin. The rDNA is the location of the genes that encode the ribosomal RNAs, the RNA Polymerase (Pol) I transcribed 35S rRNA gene and the Pol III transcribed 5S rRNA gene. Regulation and maintenance of the rDNA array is important to the health of a cell because ribosomes are responsible for protein synthesis. The 150-200 tandem repeats of the rRNA genes are particularly susceptible to high rates of homologous recombination because of the repeated nature of the locus. Silent chromatin at the rDNA represses recombination and transcription by Pol II despite the high levels of transcription by Pol I and Pol III that are needed to keep up with the ribosomal needs of the cell. Loss of silent chromatin at the rDNA leads to a shortened replicative lifespan and genomic instability. Work presented in this dissertation aims to gain a better understanding of the regulation of silent chromatin at the rDNA array by analyzing individual rDNA repeat units and the important silencing proteins Sir2 and Set1.

5.2 A TETRACYCLINE REGULATABLE SYSTEM TO CONTROL Sir2

The NAD-dependent histone deacetylase Sir2 is a member of the Silent Information Regulator (SIR) family of proteins that is conserved through eukaryotes [30, 32, 34]. Sir2 is required for silent chromatin at the rDNA array in *S. cerevisiae* [26-28].

Loss of Sir2 results in increased Pol II transcription of reporter genes integrated into the rDNA and non-coding RNAs from the NTS regions [18]. In addition, homologous recombination is drastically increased in *sir2Δ* cells, resulting in destabilization of the rDNA array [29].

Marking a single rDNA repeat unit with a Pol II transcribed gene is a common approach to studying rDNA silent chromatin because reporter transcript is a measure of the strength of silencing within the marked rDNA repeat unit. However, the increased recombination in *sir2Δ* cells results in increased movement or loss of the reporter gene, making it difficult to study an individual repeat unit in these cells. A method to quickly control Sir2 levels in cells would make it possible to study the loss and re-establishment of silent chromatin as Sir2 depletes and is re-expressed before the rDNA is drastically rearranged by increased recombination when Sir2 is absent.

A tetracycline regulatable (Tet-Off) system is being developed to control the *SIR2* gene, so that the expression of the Sir2 protein can be controlled by the addition or removal of doxycycline. The endogenous Sir2 promoter was replaced with copies of the bacterial *tet* operator, and an activator fused to the *E. coli* Tn10 Tet repressor DNA binding domain (TetR_{DBD}) was integrated into the genome. With this two-part genetic system, *SIR2* transcription can be stopped by the addition of doxycycline to growth media, and *SIR2* can be expressed again after removal of doxycycline, thereby controlling the level of Sir2 protein in the cell.

Sir2 is limiting for rDNA silent chromatin; if Sir2 is overexpressed the strength of silent chromatin increases, if Sir2 is underexpressed silent chromatin at the rDNA is

reduced [36]. Therefore, the Tet-Off system must express Sir2 at the same level as the endogenous promoter so that rDNA silent chromatin remains in the wild type state in the absence of doxycycline. Replacing the endogenous *SIR2* promoter with two *tet* operators that are controlled by the activation domain of Gcn4 fused to the TetR_{DBD} results in a level of Sir2 in the cell that is close to endogenous. However, expression of Sir2 is not stable throughout the growth of a culture; Sir2 levels begin to decrease after five hours of growth without doxycycline. In addition, after the removal of doxycycline Sir2 is not detectable by Western blot until after extended growth.

These expression issues are problematic if this system is to be used to study the re-establishment of silent chromatin. It is suspected that expression of the Gcn4-TetR_{DBD} is causing the unexpected decrease in Sir2 expression; if Gcn4-TetR_{DBD} is not constitutively expressed, *SIR2* will not be re-expressed. To address this, the CMV promoter driving expression of the Gcn4-TetR_{DBD} will be replaced with a constitutive promoter from *S. cerevisiae*, such as *ADHI*. This modification should optimize the Tet-Off system for regulating Sir2 expression and will be used in future experiments to investigate the establishment of rDNA silent chromatin.

5.3 DISCOVERY OF rDNA POSITION EFFECT

Each of the 150-200 copies of the rDNA repeat unit has the same DNA sequence, therefore it has been assumed that silent chromatin within each rDNA repeat is the same across the rDNA array. To test this assumption, a collection of strains was made that had a single Pol II-transcribed *HIS3* reporter gene integrated into an rDNA repeat at the

same position within an rDNA repeat but in a different rDNA repeat unit within the rDNA array. In a collection of sixteen yeast strains, the strength of silent chromatin in the *HIS3*-marked repeat was determined by measuring the level of *HIS3* transcript. It was found that the level of *HIS3* transcript varies among rDNA repeat units. These sixteen yeast strains were also assessed for the rate of mitotic recombination by measuring the loss of the *HIS3* gene during non-selective growth. It was found that the rate of loss of the *HIS3* gene varied, and that a higher rate of loss of *HIS3* was found in yeast strains with the highest level of *HIS3* transcript. These data suggest that silent chromatin across the rDNA array is not equally capable of repressing Pol II transcription and mitotic recombination. This conclusion was further supported by additional yeast strain collections. Silent chromatin varies in its ability to silence *HIS3* when it is integrated into another region of an rDNA repeat (NTS1), when the direction of *HIS3* transcription is reversed, and when *HIS3* is replaced by the *S. cerevisiae TRP1* gene, suggesting that variation in silencing is not specific to *HIS3*. These observations indicate that rDNA silent chromatin is not equivalent in each rDNA repeat, a property of rDNA silent chromatin being called rDNA Position Effect (rDNA-PE).

To gain insight into the mechanism behind rDNA-PE, experiments to investigate the role of modified histones in rDNA-PE were performed. Set1, the histone methyltransferase responsible for mono-, di- and tri-methylation of the fourth lysine residue on the tail of histone H3 (H3K4) was deleted in four strains with *HIS3* in NTS2. Analysis of *HIS3* transcript in *set1Δ* cells by Northern blot revealed variation in the strength of silent chromatin, suggesting rDNA-PE is not controlled by Set1. In strain

pairs that had *HIS3* in the same rDNA repeat unit in *SET1*⁺ and *set1*Δ cells, a comparison could be made to determine the fold-loss of silencing. In three of the four strains in which this comparison could be made, *set1*Δ cells showed a 1.47 to 2.57-fold increase in *HIS3* transcript. These data are in agreement with a previous report that loss of Set1 causes a three-fold reduction in Pol II transcriptional silencing [35]. Interestingly, one *set1*Δ strain showed an increase in Pol II silencing compared to the *SET1*⁺ parent. This unexpected result suggests that the role of Set1 in silent chromatin may vary between rDNA repeat units.

The histone deacetylase Sir2 is required for rDNA silent chromatin. Because the strength of silent chromatin changes as Sir2 levels change in the cell, it was hypothesized that Sir2 association would vary among rDNA repeats, correlating with the strength of Pol II silencing. That is, repeats that are very silent will have high levels of Sir2 association, whereas repeats that are less silent will have less Sir2. Chromatin immunoprecipitation (ChIP) experiments with Sir2-specific antibody were performed and the resulting DNA was analyzed by quantitative PCR. It was found that association of Sir2 does in fact vary among rDNA repeat units that have *HIS3* in NTS2. The least silent repeat had the lowest level of Sir2 associated with the *HIS3* promoter, while the most silent rDNA repeat had more Sir2 associated with the *HIS3* promoter. Sir2 is a histone deacetylase, therefore ChIP experiments using antibody specific for acetylated H3K9 and H3K14 were performed. In support of the Sir2 ChIP data, the yeast strain with less Sir2 had higher levels of acetylated H3K9 and H3K14 than strains with more Sir2 at the *HIS3* promoter.

ChIP experiments with Sir2-specific antibody were also performed with four yeast strains with *HIS3* in NTS1. Sir2 association was higher in two more silent strains than in one less silent strain tested. However, a second strain that was less silent had the same amount of Sir2 associated with the *HIS3* promoter as the two more silent strains, which does not support the hypothesis that Sir2 is regulating rDNA-PE. This result suggests that regulation of rDNA-PE in NTS1 may be more complicated than in NTS2 where it appears Sir2 is the main contributor to rDNA-PE. This is not entirely unexpected; while NTS1 and NTS2 do share some silencing proteins, other proteins have been identified as important for silencing in NTS1 that do not effect silencing in NTS2.

To gain more insight into the role of Sir2 in rDNA silencing, the Tet-Off system described above (Section 5.2) was utilized. After depleting Sir2, cells were single colony purified and allowed to grow on rich solid medium without doxycycline. During this recovery growth, Sir2 was allowed to re-accumulate and rDNA silent chromatin was re-established. The *HIS3* transcript level in cells prior to doxycycline treatment was compared to the *HIS3* transcript level in cells after removal of doxycycline. Surprisingly, silent chromatin was similar before and after dox treatment. No statistically significant differences were observed between cells of the same strain before and after dox treatment. These results suggest the strength of rDNA silent chromatin is determined independently of Sir2. That is, the strength of rDNA silent chromatin within an rDNA repeat is remembered in the absence of Sir2, so that when Sir2 returns the strength of silent chromatin within an rDNA repeat also returns.

Taken together, the data support the model that Sir2 is a major contributor to rDNA-PE, but that other factors also determine the strength of silencing in NTS1. What determines the amount of Sir2 that associates with a particular rDNA repeat unit is yet to be discovered. Experiments with the Tet-Off system suggest rDNA-PE is not a random occurrence, because the strength of silencing is stable after the loss and re-establishment of Sir2. One possible model for how Sir2 association varies with rDNA repeat units is tethering of the rDNA to the inner nuclear membrane (Figure 5-1). rDNA repeat units that are tethered may stabilize the interaction of Sir2 with the rDNA, increasing the strength of silencing within the tethered rDNA repeats. In contrast, rDNA repeats that are not tethered to the inner nuclear membrane would be less silent if the interaction of Sir2 with the rDNA is not as stable. Additionally, tethering of rDNA repeats may form a three-dimensional structure that promotes stability of Sir2 association with the tethered rDNA repeats. Given that destabilization of the rDNA results in dispersed recombination foci as detected with a fluorescently marked Rad52, it is reasonable that tethering of the rDNA and the resulting three-dimensional structure could be affecting the localization or stability of proteins that associate with the rDNA array.

5.4 A NEW ROLE FOR Set1

As a new method to screen for mutants of the Set1 methyltransferase, growth assay spot tests were done using yeast strains with *HIS3* in the rDNA array and Set1 mutants. Media lacking histidine containing 3-Amino-1,2,4-triazole (3-AT), a

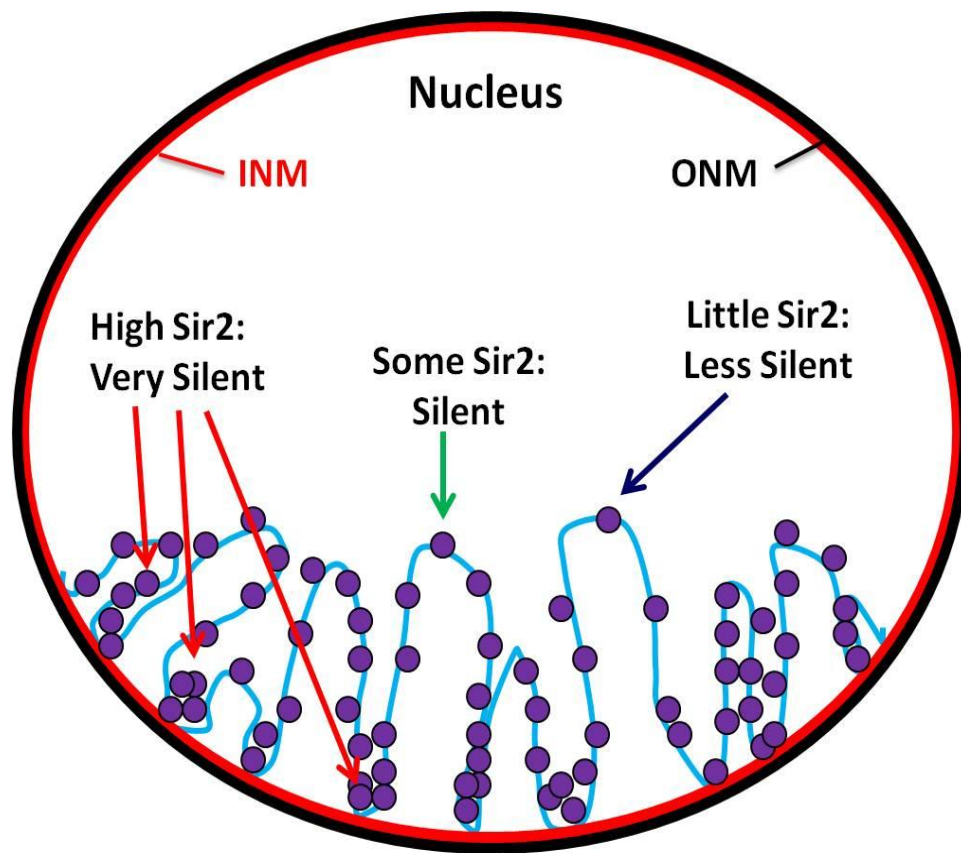


Figure 5-1. Proposed model that three-dimensional structure of the rDNA array affects association of Sir2 with rDNA repeat units. rDNA repeat units tethered to the inner nuclear membrane (INM) have more Sir2 association than rDNA repeat units further from the points of tethering. Black outline, outer nuclear membrane (ONM). Red outline, inner nuclear membrane (INM). Blue line, rDNA array. Purple circles, Sir2 protein.

competitive inhibitor of the *HIS3* gene product, was used. It was expected that strains lacking Set1 function, which have reduced rDNA silencing, would grow better than yeast strains retaining Set1 function that have rDNA silencing. Surprisingly, cells without Set1 function grew poorly, less than cells with Set1 function. Movement of the *HIS3* marker to a euchromatic region of chr *XII* and to the endogenous location of *HIS3* on chr *XV* showed that cells lacking Set1 function are sensitive to 3-AT. Northern blot analysis of *HIS3* transcript shows that cells lacking Set1 cannot induce *HIS3* transcription to the level seen in cells with Set1.

Preliminary data using yeast strains with H3K4 mutated to arginine (H3K4R), a residue that cannot be methylated by Set1, indicate that lack of H3K4 methylation is the cause of 3-AT sensitivity. These data must be confirmed in future growth assay spot tests and by Northern blot. In addition, future experiments may analyze the possible role of H3K4 methylation in regulating transcription of other genes induced by 3-AT, such as *ARG1*, to determine if 3-AT sensitivity is caused solely by a lack of *HIS3* induction or if there is a role for H3K4 methylation in the induction of other genes under these growth conditions.

5.5 SUMMARY

The collection of experiments in this dissertation has added to the foundation of knowledge of silent chromatin at the ribosomal DNA locus of *S. cerevisiae*. A Tet-Off system will be used in future experiments to gain further insight into the role of Sir2 in formation of rDNA chromatin. A previously unappreciated aspect of rDNA silent

chromatin, rDNA Position Effect, was discovered; it was shown that rDNA silent chromatin can be regulated in a repeat-specific manner. Finally, evidence was presented suggesting methylation of H3K4 by Set1 may have a role in the ability of cells to grow in histidine starvation conditions induced by the presence of 3-AT, and that this function of Set1 is not related to rDNA silencing. Together, these findings have contributed new, important information to the field of epigenetics.

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